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(54) Title: HUMAN DISEASE MODELING USING SOMATIC GENE TRANSFER
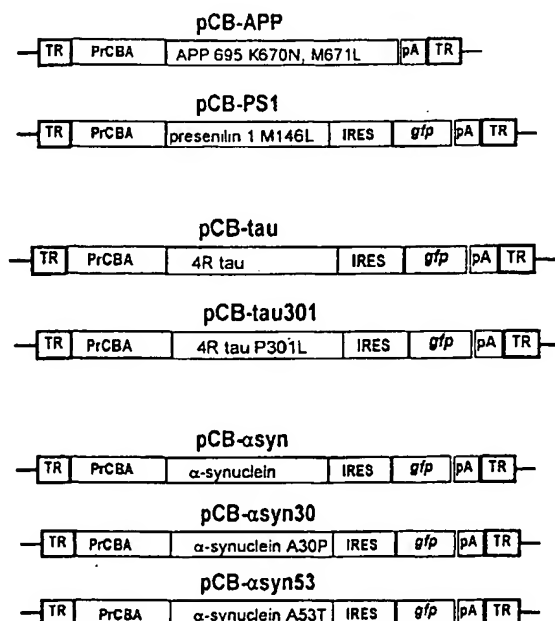
(57) Abstract: The invention provides a system for modeling neurodegenerative and other diseases through somatic gene transfer. In addition, methods of multiple gene transfer, disease analysis and drug testing are provided for.

DNA Constructs used in this application. The schematic diagrams represent the expression cassettes which are packaged into the recombinant adeno-associated virus (AAV) vectors. Abbreviations: TR, AAV terminal repeats; PrCBA, cytomegalovirus/chicken beta-actin hybrid promoter; IRES, internal ribosome entry sequence which allows for bicistronic expression of two transgenes; gfp, green fluorescent protein; pA, poly adenylation sequence. Human DNA sequences to model neurodegenerative diseases: APP, amyloid precursor protein mutant form linked to Alzheimer's disease; presenilin 1 mutant form linked to Alzheimer's disease, tau wild type and mutant form linked to fronto-temporal dementia with parkinsonism linked to chromosome 17; alpha-synuclein wild type and mutant forms linked to Parkinson's disease.

WO 02/063951 A2

TITLE OF THE INVENTION

HUMAN DISEASE MODELING USING SOMATIC GENE TRANSFER

FIELD OF THE INVENTION

5

This invention provides a system for modeling neurodegenerative and other diseases through somatic gene transfer. In addition, methods of multiple gene transfer, disease analysis and drug testing are provided for.

10   BACKGROUND TO THE INVENTION

Numerous methods of gene transfer are known in the art, and are not reviewed in any great detail here. Suffice it to say that in general, methods of gene transfer *in vitro* are well known and have been practiced for several decades. Methods of *in vivo* gene

15   transfer are much more recent, but have been successfully applied in such contexts as gene therapy efforts to overcome genetic disorders, and in disease modeling efforts, such as the production of germ-line transgenic animal models, such as gene knockout mice or transgenic mice and other animals expressing heterologous genes. For a global review of Parkinsons and other Neurodegenerative Disorders see

20   Neurodegenerative Dementias:Clinical Features and Pathological Mechanisms, (edited by Christopher Clark and John Trojanowski), McGraw-Hill (2000); and Mitochondrial Inhibitors and Neurodegenerative Disorders, (edited by Paul Sandberg et al.) Humana Press (2000).

25   In general, the known methods of *in* vivo gene transfer involve the knockout of single genes present in the genome of an animal model, or the inclusion in the germ-line of a specific transgene in the genome of an animal model. The limitations to such methods include the possibility of inducing terminal illnesses in the animal models, such that either non-viable fetuses are produced, or limited life-span animals are

30   produced. In addition, the effects of multiple gene knockouts or transgenes are extremely difficult to simulate in such systems, due to the complex temporal, gene regulatory and interaction effects in such systems. Furthermore, the germ-line transgenic models currently available tend to provide data on a very slow time scale, and such efforts as drug modeling and disease analysis are delayed by the time-scale

of transgenic animal maturation. Accordingly, there remains a need in the art for techniques which address and overcome these limitations. This invention is directed to resolving many, if not all, of these limitations in the art.

5   For example, Parkinson's disease (PD) is caused by the loss of dopamine-producing neurons in the substantia nigra (SN) and their axons that project to the striatum. Surviving neurons are characterized by two types of Lewy pathology: Lewy bodies and dystrophic Lewy neurites (see Dickson, 1999 and Trojanowski and Lee, 1999 for review). Rare genetic forms of this disease are associated with mutations in α-

10   synuclein (α-syn), a protein that is found in Lewy bodies. Two mutations in α-syn have been linked to familial PD, A53T (Polymeropoulos *et al.*, 1997) and A30P (Krüger *et al.*, 1998). The mechanisms whereby these mutations render individuals susceptible to PD remain unclear and the subject of intensive research efforts, as it is likely to provide insights into the pathophysiology of idiopathic PD as well.

15

Several models have been developed to study the effects of transgenic α-syn expression in rodents or flies. Interestingly, while the fly model mimics many cardinal features of PD, including age-dependent loss of dopamine neurons, Lewy-like inclusion bodies, and motor deficits (Feany and Bender, 2000), mammalian

20   models have been less successful or consistent. Of five rodent studies demonstrating mutant α-syn transgenic expression in neuronal cytoplasm, for example, three have found Lewy-like neurites (Masliah *et al.*, 2000; van der Putten *et al.*, 2000; Kahle *et al.*, 2000), two have found motor impairments (Masliah *et* al., 2000; van der Putten *et al.*, 2000), only one loss of dopaminergic striatal nerve terminals (Masliah *et al.*,

25   2000), and none has observed loss of dopamine perikarya in the SN. Although two of the transgenic lines effectively targeted the SN by using the tyrosine hydroxylase (TH) promoter (Rathke-Hartlieb *et al.*, 2001; Matsuoka *et al.*, in press), others were less successful in producing robust expression of α-syn in the SN (Masliah *et al.*, 2000; van der Putten *et al.*, 2000; Kahle *et al.*, 2000). These differences in the

30   expression and effects of α-syn in transgenic rodent brains have made it difficult to infer to what extent the A30P and A53T variants of α-syn are gain of toxicity mutations in SN dopamine neurons.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows examples of DNA constructs utilized according to the present

5   invention. The figure shows schematic representations of expression cassettes which are packaged into recombinant adeno-associated virus (AAV) vectors; abbreviations: TR, AAV terminal repeats; PrCBA, cytomegalovirus/chicken beta-actin hybrid promoter; IRES, internal ribosome entry sequence which allows for bicistronic expression of two transgenes; gfp, green fluorescent protein; pA, poly adenylation

10   sequence. Human DNA sequences to model neurodegenerative diseases: APP, amyloid precursor protein mutant form linked to Alzheimer's disease; presenilin 1 mutant form linked to Alzheimer's disease; tau wild type and mutant form linked to fronto-temporal dementia with Parkinsonism linked to chromosome 17; alpha-synuclein wild type and mutant forms linked to Parkinson's disease.

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Figure 2, top panel, shows neurons that are expressing transgenic APP, while the bottom panel shows neurons that are expressing transgenic PS-1 in the hippocampus region.

20   Figure 3, panels 3A to 3L, show the expression of somatically introduced transgenic tau. Figures 3A and 3B show the hippocampus region, with filamentous structures characteristic of this protein in neurons also being evident. Examples were found of an extracellular tau-immunoreactive deposit, about the size of a neuronal soma, in the toroidal shape reminiscent of the "ghost tangle" of Alzheimer's disease. This figure

25   further shows that human tau gene transfer through injection of the human four microtubule binding domain repeat P30 1L tau vector (1×10^{10} particles in 2 μl injected 3 months earlier) led to robust expression of human tau in septal neurons of the basal forebrain. Fig. 3C Shows low-magnification of the injected area, near the midline in the septal nucleus and diagonal band. Tau immunoreactivity was produced

30   along the injection, mainly on the left side of Fig. 3C. The right edge of Fig. 3C shows surrounding, non-transduced tissue. The monoclonal antibody was specific for human tau and did not produce endogenous staining in the rat tissue. Fig. 3D shows higher magnification of the transduced cells showing somatodendritic accumulation of tau immunostaining that resemble flame-shaped neurofibrillary

tangles. Higher magnification of tau accumulation in a medial septal neuron (inset). Fig. 3E is a confocal micrograph showing higher magnification of a neuron stained with the tau antibody where immunoreactive filaments are observed. This figure demonstrates that somatic gene transfer can increase tau expression and damage

5 neurons in a manner seen in a variety of neurological disorders which encompass pathological deposits of tau, such as Alzheimer's disease, fronto-temporal dementia with Parkinsonism linked to chromosome 17, amyotropic lateral sclerosis, Down's syndrome, Hallervorden-Spatz disease, Jakob-Creutzfeldt disease, multiple system atrophy, Pick's disease, and others. Figure 3 further shows the expression of P301L

10 tau, and that expression resulted in tau aggregation in neuronal cell bodies and dendrites of the adult rat basal forebrain. Figs. 3F and 3G show confocal imaging of fluorescently labeled tau (red) and bicistronic GFP native fluorescence, 2 months after tau vector gene transfer into the septum. Tau expression was somatodendritic as well as axonal, and punctate in places. Figs. 3H and 3I show that at 6 months after gene

15 transfer, a polyclonal antibody against neurofibrillary tangles labeled cell bodies in a pattern similar to the tau immunoreactivity. Figs. 3J, 3K, and 3L show that at 6 months after gene transfer, a monoclonal antibody against paired helical filament tau labeled apparent neuritic tauopathy in the basal forebrain. This antibody recognizes the epitope containing phosphorylated serine 212 and phosphorylated threonine 214.

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Figure 4 shows data from groups of untreated or vector-injected rats tested for passive avoidance behavior over a 24 hour interval, four months after the injection of the indicated vector constructs into the septum and hippocampus. Animals received a brief (0.5 seconds) small (0.8 mA) foot shock at time 0 immediately after entering a

25 dark room (training interval); their latency to enter the room 24 hours later was indicative of memory (testing interval). No difference was observed in the training intervals, a measure of locomotor activity (not shown). App, amyloid precursor protein; PS1, presenilin 1; IL6, interleukin 6; and tau protein. Each value is the mean $\pm$ SEM of the number of animals noted in parentheses. *$p < 0.05$ compared to either

30 control group (rank order test).

Figure 5 shows amphetamine stimulated locomotor activity of animals that received either the GFP control vector (UF12) or the human A30P alpha-syn vector unilaterally in the substantia nigra. At 3 months after injection of a fixed dose of AAV (1×10^{10}

particles), and 20 minutes following a 2 mg/kg administration of amphetamine challenge, alpha-syn animals were found to be 40% more active (total distance traveled in cm) over the 28 minute sessions. Two-way repeated measures ANOVA showed a main effect of the vector group (F[1,10]=6.52, P=0.029) and no effect of

5   trial interval and no significant interaction. Post-hoc tests (Scheffe and Bonferonni/Dunn) showed significant group differences, P,0.01 for each test; n=6/group. Group differences in the means during the 2nd, 4th, 5th, 6th, trial intervals; *P,0.05, t-test. This figure demonstrates that the cellular effects of somatic gene transfer on synuclein expression are manifested by behavioral changes (locomotor

10   activity) consistent with neurological damage – in this case, increased distance traveled during normal sleeping hours for the rat (roaming phenomenon). That is to say, eight months after transfer of alpha-synuclein for motor coordination on a rotating rod; these animals were deficient in this activity measure, similar to other animal models for Parkinson's disease that are not genetic in nature.

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FIG. 6 shows GFP and human α-syn expression in the SN pars compacta. (A) GFP native fluorescence after injecting the control vector, pTR-UF12 AAV, 1 month earlier at a dose of 2×10^{10} particles. Bar = 50 μm. GFP fluorescence appeared similar with this CBA promoter vector at intervals of 3, 6, and 12 months after gene

20   transfer. (B) Immunofluorescent labeling for α-syn in red, on a section adjacent to A. Immunoreactivity for α-syn was found in the gray matter of the SN pars reticulata in a similar pattern in the control vector group as in uninjected subjects, although no cellular structures were detected. (C) Immunoreactivity for α-syn in the SN pars compacta 1 month after injecting the α-syn vector, pSyn30 AAV at a dose of 2×10^{10}

25   particles, in which case perikaryal and neuritic structures were found. (D) Similar injection as in C, at an interval of 12 months after gene transfer. Inset, magnified SN neuron immunoreactive for α-syn. A-D, same magnification. (E) SN neuron expressing α-syn immunoreactivity one year post-injection as in D. Bar = 10 μm. (F) α-syn immunoreactivity (peroxidase staining in black) six months post-injection of

30   the α-syn vector. Bar = 12 μm.

Fig. 7 shows GFP and α-syn expression in striatal axons following SN injections. (A)

The control vector led to filling of striatal axons with GFP fluorescence. Bar = 240

μm. (B) Higher magnification of control vector-transduced axons expressing GFP

fluorescence, showing uniform thickness. Bar = 60 μm. (C) The α-syn vector

5   produced a distinct morphological pattern of transduced axons. Spherical varicosities

expressing GFP were always observed in the striatum after α-syn gene transfer but

not with the control vector as shown in A & B. (D) Higher magnification of

dystrophic striatal axons after α-syn gene transfer. (E) Immunoreactivity for α-syn in

red shows striatal axon structure, which was never observed in the control vector

10   group or in uninjected subjects, which showed only neuropil staining in striatal gray

matter. While the density of striatal axons expressing α-syn was similar to that of the

GFP-expressing axons in the control group, the morphology was different than the

control in A, the α-syn expressing axons had loci of dense aggregates of

immunoreactivity. (F) Higher magnification of α-syn expression in striatal

15   projections. (G) Viewing both the α-syn immunoreactivity in red with the

bicistronically expressed GFP fluorescence showed spheroids expressing both

transgenes (yellow/orange loci). (H) Higher magnification of α-syn expressing fibers

and α-syn/GFP-expressing spheroids. A-H, 6 months after gene transfer. A, C, E, G;

B, D, F, H, same magnification. It is noted that the staining in Fig. 8G and Fig. 8H

20   are best observed in a color version. Such color version may be observed in the

scientific paper, Dopaminergic Cell Loss Induced By Human A30p Alpha-Synuclein

Gene Transfer To The Rat Substantia Nigra, Ronald L. Klein, Michael A. King, Mary

E. Hamby, and Edwin M. Meyer, Human Gene Therapy, Vol. 13, No. 5.

Fig. 8 shows tyrosine hydroxylase immunoreactivity in the substantia nigra. (A) Section from an uninjected subject that was age-matched to B & C. Bar = 80 μm. (B) Section from the control vector group, 12 months after gene transfer, which appeared similar to uninjected subjects. (C) Section from the α-syn vector group, 12 months after gene transfer, showing diminished staining. This section is adjacent to the one shown in Fig. 1D. The images chosen contained the greatest density of TH-immunoreactive neurons per section for each of the 3 subjects. Unbiased stereological estimates of the TH-immunoreactive neuron profiles demonstrated 53% fewer cells in the α-syn vector group compared to the control vector group ($p<0.0005$).

SUMMARY OF THE INVENTION

This invention provides a system for modeling neurodegenerative and other diseases through somatic gene transfer. In addition, methods of multiple gene transfer, disease analysis and drug testing are provided for. Advantages of the somatic gene transfer (SGT) methodology of this invention include:

a. The ability to more precisely control the location to which the genes are transferred (i.e. spatial control of gene expression);

b. The ability to more precisely analyze the temporal effects of transferred genes at specific times in the development of otherwise normal organisms (i.e. temporal control of gene expression);

c. The ability to evaluate the effects of expression of combinations of multiple transgenes, which in a germline transgenic animal would be difficult if not impossible to achieve due to diseases which might prevent the animal model from maturing to the age-appropriate state for modeling onset of a particular, complex human disease, such as Alzheimer's.

d. Reduced cost.

e. Faster method for analyzing multiple genes which contribute to complex, multifactorial neurodegenerative diseases.

f. The models can be used for drug testing against specific neurodegenerative diseases as well as for studying the pathologies themselves.

g.   The methodology provides a means to supplement existing germline
        transgenic models with additional somatically provided gene products to
        modulate the transgenic model.

h.   Additionally, another possibly unique aspect of this technology is its emphasis

5   on the creation of a disease condition in an otherwise healthy animal, as
        opposed to, say, gene therapy techniques developed to treat disease conditions,
        or germ-line based disease models in which the animal model is diseased (if
        only nacently) from the outset.

10   `   Accordingly, objects of this invention include provision of a system which meets any
        or all of the foregoing criteria. In specific embodiments of this invention, such
        diseases as Alzheimer's Disease (AD), Parkinson's Disease (PD), and Huntington's
        Disease (HD) are effectively modeled through somatic gene transfer, as opposed to
        known methods of germline transgenesis. This patent disclosure demonstrates the

15   present inventors' ability to produce brain aggregates through somatic gene transfer of
        a mutant form of human tau (P301L), known to be associated with "fronto-temporal
        dementia with Parkinson's linked to chromosome 17 (FTDP-17)", or through somatic
        gene transfer of mutant α-synuclein (A30P), known to be associated with PD. This
        patent disclosure also discloses success in somatic expression of a mutant amyloid

20   precursor protein (APP), and of a mutant presenilin-1 (PS1), mutant forms of each of
        which are known to be associated with AD.

        Specifically, the present disclosure includes critical data on cell loss in an SGT-
        treated animal model that mimics the cell loss of Parkinson's Disease. This and other

25   data, including behavioral data, indicate the effectiveness of localized SGT according
        to the present invention to develop appropriate models for neurodegenerative and
        other diseases having known or suspected relationship to genes deliverable via SGT.

        Thus, one object of the invention is to use SGT to induce a specific disease state at a

30   desired stage or age in the life of an organism, such as a laboratory test organism. A
        specific object is to use SGT to introduce genetic material that results in a laboratory
        animal model of Parkinson's Disease. Another object is to use SGT according to the
        present invention to introduce genetic material that results in a laboratory model of

other neurodegenerative diseases, including but not limited to Alzheimer's Disease and Huntington's Disease. A related objective is to provide methods to study such neurodegenerative diseases in relation to pharmacologically active substances and or . nutrient regimes that are evaluated to determine their ability to slow, reduce, reverse

5   or eliminate the adverse effects of a particular degenerative disease. A related objective is to evaluate the effects of environmental inducers in a model system according to the present invention, alone or in combination with pharmacologically active substances and or nutrient regimes to increase the understanding of the interactions among these classes of inputs.

10

Thus, a general object of the invention is to evaluate and identify appropriate treatments and/or protocols in such laboratory animal models the slow, reduce, and/or eliminate the disease state so induced by the methods of the invention. Through such studies not only is an appropriate understanding gained of the interaction of life stage

15   and age, physical and nutritional status, and effects of pharmacologically active substances, but more critically to the clinical prognosis of humans afflicted with such diseases, appropriate environmental inducers and/or dietary regimes may are identified or shown irrelevant, and appropriate pharmacologically active substances are identified for appropriate clinical testing in humans.

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An additional advantageous aspect of the subject invention pertains to combining genes, or mutant forms thereof, in trans or in cis, in known delivery vehicles (e.g. viral vectors) for simultaneous or proximal delivery to enhance the desired disease effect, or to more broadly analyze the effect of various different gene expressions.

25   For example, a "cocktail" of genetic vectors comprising differing genes could be injected, at the desired site, or could be injected separately by two or more injections. The inventors have discovered that certain cells have been susceptible to expression of certain aberrant genes while other cells are resistant to expression of the aberrant gene product. Delivery of two or more genes via SGT will provide, for the first time,

30   a means to elucidate what molecules, compounds, receptors, or cellular structures are responsible for susceptibility or resistance to certain aberrant proteins and genes.

Furthermore, the subject SGT methods taught herein may be employed in combination with known transgenic model systems (e.g. transgenic mice). For

example, SGT could be conducted on transgenic rodents expressing alpha-synuclein, SGT may also introduce alpha synuclein but preferably introduces another aberrant gene . The subject SGT methods make combining different model systems possible. Such combinations provide a more flexible and accurate investigative tool to elucidate

5   cellular mechanisms involved in neurodegenerative diseases.

These and other objects and advantages of the present invention are shown by the following detailed disclosure and examples, which are not to be taken to be limiting of the full scope of the present invention.

10

DETAILED DISCLOSURE OF THE PREFERRED EMBODIMENTS

This invention provides a system for modeling neurodegenerative and other diseases through somatic gene transfer. In addition, methods of multiple gene transfer, disease

15   analysis and drug testing are provided for. Naturally, variations on this theme, as well as other aspects of the invention and equivalents thereof are to be considered as part of the present patent disclosure.

As opposed to methods of germ-line modification of animals to produce models of

20   various neurodegenerative and other diseases, or methods of transferring genes to achieve therapeutic results, this patent disclosure is directed to methodology wherein a disease state analog is produced in an animal model via somatic gene transfer.

As used herein, the term "somatic gene transfer" or "SGT" is intended to mean the

25   process whereby a gene not normally present in an organism is transferred into that organism in a manner that does not implicate the modification of the germ-line of the recipient organism. In other words, if the recipient organism were to produce progeny, in general, the process of SGT would not result in inheritance of the transferred genes to the progeny. This is not to say that it is impossible for genes

30   transferred to an organism by SGT to be incorporated into the germ line of the recipient organism and thence transferred to progeny. Such events as viral induced gene incorporation, transposon mediated gene integration and the like, could conceivably result in the incorporation of genes transferred by SGT into a recipient organism's germ line. However, it should be understood that this is not a principal

purpose of conducting SGT. SGT may, however, be practiced according to the present invention in a recipient which already has a modified germ line. For example, a mouse having a particular gene knockout in its germ plasm may, through SGT, be induced to express one or more other genes. In this manner, it is possible to analyze

5 the effects and interplay of the given gene knockout with the genes transferred by SGT.

SGT is achieved according to the present invention by appropriately cloning genes, known or hereafter discovered, to appropriate gene regulatory signals, such that upon

10 introduction into an organism, the relevant genes introduced by SGT are transcribed and translated appropriately, to exert a biological effect. Those skilled in the art are well familiar with appropriate gene expression promoters, terminators, enhancers, vectors and the like, and this patent disclosure therefore does not review in great detail those methodologies and compositions with which those skilled in the are well

15 familiar.

In one preferred embodiment according to the present invention, SGT is achieved using appropriately constructed viral vectors. Viral vectors that may be used according to this invention include, but are not limited to, lentivirus vectors, herpes

20 virus vectors, adenovirus vectors, retroviral vectors, and equivalents thereof. One preferred viral vector system for this purpose includes the use of recombinant adeno-associated viral (AAV) vectors. AAV's are efficient, their infection is relatively long-lived and is generally non-toxic, unless a toxic transgene is recombined therein. AAV is a small, helper-dependent parvovirus consisting of a single strand 4.7 kb DNA

25 genome surrounded by a simple, non-enveloped icosahedral protein coat. Approximately 85% of the adult human population is seropositive for AAV. However, no pathology as been associated with AAV infection. Adenovirus or herpesvirus is generally required as a helper virus to establish productive infection by AAV. In the absence of helper virus, the AAV genome also amplifies in response to

30 toxic challenge, e.g. UV irradiation, hydroxyurea exposure, and the like. In the absence of either toxic challenge or helper virus, wild-type AAV integrates into human chromosome 19 site-specifically as a function of AAV Rep proteins that mediate the formation of an AAV-chromosome complex at the chromosomal integration site. Up to 96% of the viral-genome may be removed, leaving only the

two 145 base pair (bp) inverted terminal repeats (ITRs) which are sufficient for packaging and integration of the viral genome. Methods for efficient propagation of recombinant AAV, rAAV, have been developed in the art, including the use of mini-adenoviral genome plasmids, plasmids encoding AAV packaging functions and

5   adenovirus helper functions in single plasmids. Furthermore, methods of rAAV isolation have developed to the point where methods for isolation of highly purified rAAV are a relatively straightforward and rapid undertaking. Likewise for methods of titration of rAAV stocks. Use of green fluorescent protein (GFP) a well-characterized 238 amino acid fluorescent protein is frequently used in a bicistronic

10   arrangement in rAAV to trace rAAV-mediated transgene expression. Promoters for selective and specific expression of rAAV mediated gene transfer have also been identified.

Methods of making and using rAAV and delivery of rAAV to various cells *in vivo* are
15   disclosed in US Patent Publication Nos. 5,720,720; 6,027,931; 6,071,889; WO 99/61066; all of which are hereby incorporated by reference for this purpose.

With regard to methods for the successful, localized, long-term and non-toxic transgene expression in the nervous system through SGT using adeno-associated virus
20   (AAV) and selected promoters, reference is made to Klein et al, 1998, Experimental Neurology 150:183-194, "Neuron-Specific Transduction in the Rat Septohippocampal or Nigrostriatal Pathway by Recombinant Adeno-associated Virus Vectors". '

With respect to a method of gene therapy using recombinant AAV with significant
25   persistence through stable expression of the neurotrophic factors NGF or BDNF, and resultant neurochemically quantifiable therapeutic effects, reference is made to Klein et al, Neuroscience 90:815-821, " Long-term Actions of Vector-derived Nerve Growth Factor or Brain-derived Neurotrophic Factor on Choline Acetyltransferase and Trk Receptor Levels in the Adult Rat Basal Forebrain."

30
With regard to achievement of quantifiable behavioral effects through somatic transgene expression in the nervous system through AAV vectored expression of BDNF, reference is made to Klein et al, 1999, Brain Research 847:314-320,

"Prevention of 6-hydroxydopamine-induced Rotational Behavior by BDNF Somatic
Gene Transfer."

5   With respect to a review of the state of the art of germline transgenic mouse models
for neurodegenerative diseases, including a large number of references in the field of
germline transgenic mouse modeling of neurodegenerative diseases, reference is made
to Price et al, 1998, Science 282:1079-1083, "Genetic Neurodegenerative Diseases:
The Human Illness and Transgenic Models."

10   With regard to a review of the state of the art of germline transgenic mouse models
for neurodegenerative diseases, including a large number of references in the field of
germline transgenic mouse modeling of neurodegenerative diseases, reference is made
to PCT Publication WO99/61066, Published 2 December, 1999, Avigen, Inc., based
on Prior US Applications filed 5/27/98 and 5/18/99, "Convection-Enhanced Delivery
15   of AAV Vectors". However, the review does not substantively address somatic cell
transgenesis methodology.

As with the WO99/61066 publication discussed above, reference is made to US
Patent 5,720,720, "Convection-Enhanced Drug Delivery", for its disclosure of
20   methods relating to the delivery of various compounds, including viruses, to the CNS
via CED.

With respect to administration of genes to neural precursor cells induced to divide
through contact with growth factors to facilitate incorporation of the genetic material
25   into the cell progeny, reference is made to US Patent 6,071,889, "In Vivo Genetic
Modification of Growth Factor-Responsive Neural Precursor Cells." AAV mediated
gene delivery is mentioned, although the method appears to be limited to the *ex vivo*
administration of nucleic acids and growth factors to neuronal cells, and the thus
treated cells are then administered to the living organism.

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With regard to genes known in the art to which reference is made herein, amyloid
precursor protein, APP, was described by Hisao et al, 1996, "Correlative memory
deficits, AB elevation, and amyloid plaques in transgenic mice," Science 274:99-102.
Presenilin-1, PS-1, was described by Duff et al, "Increased amyloid-beta 42(43) in

brains of mice expressing mutant presenilin 1," Nature 1996, 383(6602):710-713.

Tau was described by Nacharaju et al 1999, "Accelerated filament formation from tau protein with specific FTDP-17 missense mutations," FEBS Letters 447:195-199.

Alpha-synuclein was described by Polymeropoulos et al, 1997 "Mutation in the alpha-

5　synuclein gene identified in families with Parkinson's disease," Science 276:2045-2047; and by Kruger et al., "Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease," Nat. Genet. 18(1998):106-108. All of these references are hereby incorporated by reference for their teachings of these genes.

10　Turning now to specific applications of the present invention, wherein SGT is used to induce specific disease states is an appropriate animal model, examples are provided herein of success achieved by the instant inventors in the particularly challenging area of neurodegenerative disease modeling. Those skilled in the art will appreciate that the present invention should not be restricted to the specifics of the examples

15　provided herein, because the operative principles for achieving success in the particularly challenging area of neurodegenerative diseases also apply in less challenging areas of human disease modeling, not requiring delivery of genes to the central nervous system (CNS). In addition, those skilled in the art will appreciate that the present invention is particularly useful in modeling diseases of the CNS, because

20　of the refined control that the present methodology provides in terms of the ability to specifically target selected CNS tissues of interest. In addition, advantages of this invention include:

　　　(a)　The ability to more precisely control the location to which the genes are transferred (i.e. spatial control of gene expression);

25　　(b)　The ability to more precisely analyze the temporal effects of transferred genes at specific times in the development of otherwise normal organisms (i.e. temporal control of gene expression);

　　　(c)　The ability to evaluate the effects of expression of combinations of multiple transgenes, which in a germline transgenic animal would be

30　　　　difficult if not impossible to achieve due to diseases which might prevent the animal model from maturing to the age-appropriate state for modeling onset of a particular, complex human disease, such as Alzheimer's.

　　　(d)　Reduced cost.

(e)   Faster method for analyzing multiple genes which contribute to complex, multifactorial neurodegenerative diseases.

(f)   The models can be used for drug testing against specific neurodegenerative diseases as well as for studying the pathologies themselves.

5   (g)   The methodology provides a means to supplement existing germline transgenic models with additional somatically provided gene products to modulate the transgenic model.

(h)   Additionally, another possibly unique aspect of this technology is its emphasis on the creation of a disease condition in an otherwise healthy

10   animal, as opposed to, say, gene therapy techniques developed to treat disease conditions, or germ-line based disease models in which the animal model is diseased (if only nacently) from the outset.

Accordingly, objects of this invention include provision of a system which meets any
15   or all of the foregoing criteria. In specific embodiments of this invention, such diseases as Alzheimer's Disease (AD), Parkinson's Disease (PD), and Huntington's Disease (HD) are effectively modeled through somatic gene transfer, as opposed to known methods of germline transgenesis. This patent disclosure demonstrates the present inventors' ability to produce brain aggregates through somatic gene transfer of
20   a mutant form of human tau (P301L), known to be associated with "fronto-temporal dementia with Parkinson's linked to chromosome 17 (FTDP-17)", mutant α-synuclein (A30P), known to be associated with PD. This patent disclosure also discloses success in somatic expression of a mutant amyloid precursor protein (APP), and of a mutant presenilin-1 (PS1), mutant forms of each of which are known to be associated
25   with AD. Other genes of interest with respect to practice of the methods of this invention include, but are not limited to: GAP43, interleukins, especially interleukin-6 (IL-6), gamma-secretase, and combinations thereof. Particularly preferred combinations of genes for transfer to an animal model in accordance with the methodology of this invention include, but are not limited to: APP in combination
30   with presenilin; APP in combination with presenilin plus tau; APP in combination with presenilin plus tau plus IL6; combinations, permutations and variations thereof.

Mutations in the genes for tau and alpha-synuclein can result in abnormal protein deposition, formation of neurofibrillary tangles and Lewy bodies, and death of specific neuron populations. For example, splice site and mis-sense mutations in the tau gene are found in families of neurofibrillary pathology like frontotemporal

5   dementia with Parkinsonism linked to chromosome 17. Transgenic models of neurodegeneration provide functional genomic information about the impact of inherited mutations. Accordingly, somatic cell transgenic models of neurodegeneration are useful for functional genomic studies at particular time points in the lifespan and in particular brain regions. In addition to providing spatio-

10   temporal control of transgene expression, the adeno-associated viral (AAV) vector system enables mixed gene combinations, which are important for complex neurological diseases. Many of these mutant genes are by now well known in the art, having been cloned sequenced and extensively characterized. Accordingly, those skilled in the art, based on the instant disclosure, would be fully enabled to practice

15   the present methods of SGT using such genes known in the art, as well as genes hereafter identified as playing potential roles in development of human neurodegenerative, as well as other human diseases. As a result, the methods disclosed herein provide versatile systems for modeling human diseases, as well as various veterinary diseases, in a rapid, efficient manner, which does not require the

20   delay and complexity of germline disease modeling.

According to the present disclosure, certain specific nucleic acid vector constructs are disclosed by way of exemplary support. Reference is made to figure 1 herein, which shows DNA constructs which may be used according to the methods of this invention.

25   Those skilled in the art will appreciate that, based on this disclosure, a wide variety of disease-causing genes, transcriptional promoters, translational regulators, effectors, initiators, cis and trans acting elements, enhancers, marker genes, and the like may be employed according to the methods disclosed herein, without departing from the heart of this invention, namely the induction of disease states in an appropriate animal

30   model through somatic transfer of expressible gene constructs. Those skilled in the art will further appreciate, based on the present disclosure that the methods disclosed · herein are also applicable to such models of disease states where a germline modification has been made, and somatic gene transfer is accomplished in the genetic background of an already altered germline in order to elucidate such effects as

masking of one allele by another, synergistic effects between different defective alleles, gene knockouts, and the like.

EXAMPLE 1

5   ## INDUCTION OF TAUOPATHY IN ANIMAL MODELS:

The present inventors have expressed a mutant form of human tau (P301L) using an AAV vector system in the septal nucleus of the basal forebrain and the hippocampus in the adult rat. The vector-derived tau accumulated in cell bodies and dendrites and

10   formed aggregates as observed by co-localization with the reporter gene, green fluorescent protein (GFP), which was bicistronically expressed by the vector (i.e., GFP filled neurons and tau distribution within cell bodies was clustered). The neurofibrillary pathology observed in this model shows abnormal accumulation of tau in neuron cell bodies and dendrites, filaments immunoreactive for

15   hyperphosphyorylated tau, neuritic immunoreactivity for several antibodies that recognized neurofibrillary tangles in Alzheimer's and FTDP-17, and a dramatic induction of reactive astrogliosis. See figures 2 and 3 provided herewith and the description thereof provided hereinabove.

20   ## EXAMPLE 2

SGT AS A METHOD FOR SUPPLEMENTING GERMLINE ANIMAL MODELS:

Another utility of the present vector system is to apply genes in *trans* to existing germline mouse and other animal models, for example, by expressing tau in current

25   models of amyloidosis to introduce tangles.

EXAMPLE 3

INDUCTION OF PARKINSON'S DISEASE ASSOCIATED CNS LESIONS IN ANIMAL MODELS:

30

A gene linked to autosomal dominant Parkinson's disease, alpha-synuclein, harboring the A30P mutation, was expressed in the rat substantia nigra. Transduced neurons in this area had aggregates rich in alpha-synuclein and axons with large varicosities (5-10 micrometers in diameter) that were not found in control vector samples.

Overexpression of alpha-synuclein in the nigrostriatial pathway also elevated rates of amphetamine-stimulated locomotor behavior, which is apparently consistent with reduced locomotor response in alpha-synuclein knockout mice (Abeliovich et al., 2000). Accordingly, it is concluded that the somatic transgenic models disclosed

5   herein are useful for studying mechanisms of neurodegenerative disease pathogenesis as well as gene structure-function relationships of tau and alpha-synuclein.

EXAMPLE 4
PARKINSON'S DISEASE ASSOCIATED CNS LESIONS IN ANIMAL MODELS USING SGT OF THIS INVENTION INDUCE SIMILAR BEHAVIORAL AND MORPHOLOGIC LESIONS TO THOSE FOUND IN GERMLINE TRANSGENIC ANIMAL MODELS

10

Through practice of the SGT method of this invention, we have found in connection

15   with Parkinson's Disease that alpha synuclein overexpression causes a behavioral change in locomotor activity associated with dopamine transmission. In addition, we have found that cellular processes are essentially identical to those seen in standard germline transgenic animal models (see figure 2 provided herein) produced using alpha synuclein expression. See figure 4 and the description thereof provided

20   hereinabove.

EXAMPLE 5
ALZHEIMER'S DISEASE ASSOCIATED CNS LESIONS IN ANIMAL MODELS USING SGT OF THIS INVENTION INDUCE SIMILAR BEHAVIORAL AND MORPHOLOGIC LESIONS TO THOSE FOUND IN GERMLINE TRANSGENIC ANIMAL MODELS

25

Through practice of the SGT method of this invention, we have found in connection with Alzheimer's Disease that tau overexpression causes intracellular distribution of

30   the protein that is essentially identical to that seen in the only existing germline transgenic animal model in which neurofibrillary tangles associated with the disease are observed. The neuritic damage observed by the present inventors in using the SGT methodology of this invention is similar to that seen in germline transgenic mice overexpressing the gene.

EXAMPLE 6

ALZHEIMER'S DISEASE ASSOCIATED CNS LESIONS IN ANIMAL MODELS
USING SGT OF THIS INVENTION INDUCE SIMILAR BEHAVIORAL AND
MORPHOLOGIC LESIONS TO THOSE FOUND IN GERMLINE TRANSGENIC
5   ANIMAL MODELS

Reference is made here to figures 1 and 2 of D.W. Dickson, "Tau and alpha-synuclein
and their role in neuropathology, Brain Pathology", 9:657-661 (1991), where tau and
synuclein immunohistochemistries are shown, which demonstrate some of the
10   neuropathological processes associated with age-related disease states such as
Alzheimer's, Picks, and Parkinson's diseases. It is noted that cells appear to be filled
with gene products that appear to be overproduced in such diseases. Notable types of
aberrant gene products include tau, amyloid, and synuclein.

15   Figures 3 and 6 hereof show similar types of aberrant gene expression for tau and
synuclein, respectively. In each case, a similar procedure was used: a single injection
of a vector was used to introduce the relevant gene into an appropriate brain region,
followed by monitoring over sufficient intervals of up to several years for the relevant
pathological morphology to develop.
20

In Figure 3, plates C, D, and E are shown wherein tau protein aggregation induced by
SGT of tau is demonstrated. In plate C, two months after injection of the human 4R
P301L tau vector (1×10^9 particles), tau immunoreactivity was found in cell bodies
localized to the injection site in the medial septal/diagonal band area. The right side
25   of the panel shows the non-transduced, surrounding tissue. In plate D, higher
magnification of rat septal neurons expressing vector-derived human tau is shown.
Some of the transduced neurons, like that shown in the inset, showed densely stained
tau in the flame-like shape of neurofibrillary tangles. In plate E, co-localization of tau
(stained with Texas red) and GFP (native fluorescence) is shown. This vector
30   produces both tau and GFP bicistronically. While GFP filled the neurons and the
nucleus, tau intensely accumulated in cell bodies, but not the nucleus. The filter set
captured both red (tau) and green (GFP) fluorescence. This figure demonstrates that
somatic gene transfer can increase tau expression and damage neurons in a manner
seen in a variety of neurological disorders which encompass pathological deposits of

tau, such as Alzheimer's disease, fronto-temporal dementia with Parkinsonism linked to chromosome 17, amyotropic lateral sclerosis, Down's syndrome, Hallervorden-Spatz disease, Jakob-Creutzfeldt disease, multiple system atrophy, Pick's disease, and others.

5

Furthermore, Figure 3F-3L shows the expression of P301L tau, and that expression resulted in tau aggregation in neuronal cell bodies and dendrites of the adult rat basal forebrain. (F, G) Confocal imaging of fluorescently labeled tau (red) and bicistronic GFP native fluorescence, 2 months after tau vector gene transfer into the septum. Tau

10 expression was somatodendritic as well as axonal, and punctate in places. (H, I) At 6 months after gene transfer, a polyclonal antibody against neurofibrillary tangles labelled cell bodies in a pattern similar to the tau immunoreactivity. (J-L) At 6 months after gene transfer, a monoclonal antibody against paired helical filament tau labeled apparent neuritic tauopathy in the basal forebrain. This antibody recognizes

15 the epitope containing phosphorylated serine 212 and phosphorylated threonine.

Figure 6 shows the results of human alpha-synuclein gene transfer using a construct having the human A30P alpha-syn mutation. The vectors used contain bicistronic GFP expression. AAV was injected into the midbrain 6 months earlier at a dose of

20 $1x10^{10}$ particles. See explanation of this figure in Brief Description of the Drawings, above, and the discussion in Example 8, below. Overall, this figure demonstrates that somatic gene transfer can increase synuclein expression and damage neurons in a manner seen in a variety of neurological disorders at the cellular level, including Parkinson's disease, Hallervorden-Spatz disease, Lewy body dementia, multiple

25 system atrophy, Down's syndrome, and other diseases associated with this gene product.

Accordingly, these data demonstrate successful induction of human neurodegenerative disease relevant morphology. Transfer of multiple genes

30 according to this methodology results in variations and combinations of the results seen in these figures.

EXAMPLE 7

BEHAVIORAL EFFECTS OF TRANSFERRING SEVERAL GENES RELATED TO DEMENTIA INTO THE BRAIN

5   Adult male rats received intraseptal and intrahippocampal injections of a control AAV vector expressing GFP or a combination of vectors that encode amyloid precursor protein (APP), tau, IL6 and presenilin-1 (see Figure 1). Expression of all genes (control and disease-related) was driven by the CBA promoter. Four months after injections, both groups were evaluated for memory related performance according to a

10   passive avoidance paradigm, followed by an evaluation of their brains for gene expression. Another, untreated control group was included for determination of the potential toxic action of control AAV. The results shown in Figure 5 show that there was a significant reduction in latency (memory of a mild foot shock 24 hours earlier) in the group of rats receiving the multiple dementia-related gene cocktail, compared

15   to either of the other groups. The control vector had no effect on this memory related behavior.

Evaluation of brain tissues injected with the multiple vectors for the disease-related genes (APP+PS1+tau+IL6) revealed that multiple gene products were formed in the

20   hippocampus. Typical pictures showing this multiple gene expression from the same injected brain, for example, are shown in Figures 2 and 3. Figure 2, top panel, shows neurons that are expression transgenic APP, while the bottom panel shows neurons expressing transgenic PS-1 in the same brain region. Figure 3A and 3B show the expression of transgenic tau in this region, with filamentous structures characteristic

25   of this protein in neurons. Examples were found of an extracellular tau-immunoreactive deposit, about the size of a neuronal soma, in the toroidal shape reminiscent of the "ghost tangle" of Alzheimer's disease. These structures are believed to form when neurons with neurofibrillary tangles encircling the nucleus die; after all of the debris is removed, the insoluble tangle leaves a ring. A zone of

30   . reactive astrocytosis was observed around the injection site in the region containing GFP+ neurons. No colocalization of GFP and GFAP (marker for glial cells, not neurons) was observed, although adjacent GFP+ neurons and immunolabelled astrocytes were common, indicating that this vector delivery system was selective for

neurons. These observations are consistent with a pathological effect of the polygenic transfection, such as that observed in a variety of neuropathological conditions.

EXAMPLE 8

5 EFFECTS OF TRANSFERRING ALPHA-SYNUCLEIN ON LOCOMOTOR ACTIVITY, CELLULAR MORPHOLOGY AND GROSS LOSS OF DOPAMINERGIC CELLS IN THE SN

The alpha-synuclein gene has been associated with aberrant neuronal pathologies
10 including Parkinson's disease. Using the AAV vector system, this gene was transduced into the substantia nigra of adult male rats, the brain region that degenerates in Parkinson's disease. Eight months later, they were assayed for motor coordination on a rotating rod (Figure 6B). These animals were deficient in this activity measure, similar to other animal models for Parkinson's disease that are not
15 genetic in nature. That this gene was expressed in the substantia nigra was demonstrated in Figure 4H-J; neurons expressing this disease-related gene had punctate staining of their processes, similar to degenerating neurons in a variety of pathological conditions.

20 Figure 6A shows amphetamine stimulated locomotor activity of animals that received either the GFP control vector (UF12) or the human A30P alpha-syn vector unilaterally in the substantia nigra. At 3 months after injection of a fixed dose of AAV (1×10^{10} particles), and 20 minutes following a 2 mg/kg administration of amphetamine challenge, alpha-syn animals were found to be 40% more active (total distance
25 traveled in cm) over the 28 minute sessions. Two-way repeated measures ANOVA showed a main effect of the vector group ($F[1,10]=6.52$, $P=0.029$) and no effect of trial interval and no significant interaction. Post-hoc tests (Scheffe and Bonferonni/Dunn) showed significant group differences, $P,0.01$ for each test; $n=6$/group. Group differences in the means during the 2nd, 4th, 5th, 6th, trial intervals;
30 *$P,0.05$, t-test. This figure demonstrates that the cellular effects of somatic gene transfer on synuclein expression are manifested by behavioral changes (locomotor activity) consistent with neurological damage – in this case, increased distance traveled during normal sleeping hours for the rat (roaming phenomenon).

In this example, the direct effects of α-syn transgenic expression in the SN using the adeno-associated virus (AAV) vector system in adult animals were evaluated. AAV vectors have been used to transduce brain neurons efficiently (Kaplitt *et al.*, 1994; McCown *et al.*, 1996; Mandel *et al.* 1997) without an overt pathological response for

5 at least 1 year (see Peel and Klein, 2000 and Klein *et al.*, 2000 for review). This suggests the suitability using AAV vectors for long-duration laboratory studies of progressive neurological diseases without interference from a secondary adverse effect from the presence of the AAV. While this vector system has been applied for gene therapy historically, it is also well-suited to study gene function at specific ages

10 in targeted brain regions, which proves useful to study neurodegenerative diseases that are age-dependent and associated with specific parts of the brain. Of potentially even greater significance as a useful model system to study neurodegenerative diseases, the present invention can be used in studies that include pre-treatments and/or post-treatments of pharmacologically active substances, nutrient regimes,

15 environmental inducers, or combinations of these.

As used in this invention, the term pharmacologically active substances is defined to be , and includes known or unknown chemical compounds, virus, protein, peptide, amino acid, lipid, carbohydrate, nucleic acid, nucleotide, drug, pro-drug or other

20 substance or any synthetic or natural compounds, and any combinations, mixtures, variants, analogs, or mutants thereof, that demonstrate an affect on or modulate indicators of a neurogenerative disease as set forth herein, including cell loss and behavioral pathologies. Preferably, pharmacologically active substances refer to a substance that

25 slows, reduces or eliminates the adverse effects of a degenerative neurological disease.

As used in this invention, the term nutrient regimes is defined to be any provision of a specific set of nutrient intake, whether oral or provided otherwise, so as to influence

30 the physical and metabolic status of the test organism, and particularly includes antioxidants and compounds or mixtures that provide antioxidant effects, and most particularly includes antioxidants that are known or suspected to facilely cross the blood/brain barrier.

As used in this invention, the term environmental inducers are defined to include compounds and environmental inputs that are known or suspected to aggravate, reduce the effectiveness of, or otherwise deleteriously influence the normal

5   metabolism of a mammalian organism. Environmental inducers include toxins, and particularly include known neurotoxins, and most particularly include neurotoxins shown or believed to promote, induce, or otherwise lead to a neurodegenerative condition. Examples, not meant in any way to be limiting, of the latter subclass of environmental inducers include 6-hydroxydopamine, MPTP (and its MPP+ free

10   radical), and rotenone. Environmental inducers also include known nutrients to which a test organism is exposed in excessive quantity, for instance Manganese (see generally Mechanisms of Degeneration and Protection of the Dopaminergic System, 2001 edited by Juan Segura-Aguila, Programme of Molecular and Clinical Pharmacology, FP Graham Publishing Co., Chapters 3 and 10).

15

The A30P mutation was selected because rodents normally harbor the A53T form, because it was suggested to oligomerize more readily (Conway et al. 2000), and be more pathogenic than wild-type or the A53T mutation in flies (Feany and Bender,

20   2000).

The following subsections summarize materials and methods for this example.

DNAs and transfections

25   The CBA promoter-containing plasmid, pTR-UF12, was obtained at Vector Core laboratory at the University of Florida. The plasmid contains the AAV terminal repeats (TRs), the only remaining feature (and 4%) of the wild type AAV genome. Flanked by the TRs, the expression cassette of pTR-UF12 includes the following components, in (5' to 3') order: 1) a 1.7 kb sequence containing the hybrid CMV

30   immediate early enhancer/chicken β-actin promoter/exon1/intron (Niwa et al., 1991; Daly et al., 1999); 2) the internal ribosome entry site (IRES) from poliovirus, which provides for bicistronic expression (Dirks et al. 1993); 3) green fluorescent protein (GFP) cDNA (Klein et al., 1998); 4) and the polyA tail from bovine growth hormone.

The human A30P α-syn cDNA was provided by Dr. M. Farrer and Dr. J. Hardy
(Mayo Clinic, Jacksonville, FL). The α-syn cDNA was inserted between the CBA
promoter and the IRES element of the pTR-UF12 to derive the construct pSyn30.
Plasmids were propagated in SURE cells (Stratagene) and CsCl-purified.

5

The pTR-UF12 and the pSyn30 constructs were tested for *in vitro* expression by
calcium-phosphate transfection of human embryonic kidney 293 cells. GFP
fluorescence was observed in cells transfected with either the pTR-UF12 or the
pSyn30 DNAs but not in mock-transfected cells. On GFP immunoblots, a
10   monoclonal anti-GFP antibody (Chemicon) labeled a single band at 31 kDa in cell
samples that were transfected with either pTR-UF12 or pSyn30, but not in untreated
and mock-transfected cell samples. On α-syn immunoblots, an α-syn polyclonal
antibody (NACP-98) provided by Dr. Matt Farrer (Mayo Clinic, Jacksonville, FL)
labeled a single band at 19 kDa in cell samples that were transfected with the pSyn30
15   DNA, but not in pTR-UF12-transfected, mock-transfected, or untreated 293 cells.

Vector packaging and titering

Plasmids were packaged in rAAV by the streamlined method developed by
Zolotukhin et al. (1999). Briefly, human embryonic kidney 293 cells at 70%
20   confluence were transfected by the calcium-phosphate method with an AAV terminal
repeat-containing plasmid (pTR-UF12 or pSyn30) in an equimolar ratio with the
plasmid pDG, which provides the AAV coat protein genes, and adenovirus 5 genes
necessary for helper function in packaging (Grimm *et al.*, 1998). Transfections used
twenty 15 cm dishes and 80 μg DNA per dish. Three days after, cells and media were
25   harvested and pelleted at 3000 x g. The pellet was resuspended in 60 ml of lysis
buffer (50 mM Tris pH 8.5, 150 mM NaCl) and freeze-thawed 3 times. The sample
was then incubated with 1500 units of endonuclease (Sigma) for 30 min. at 37°C.
After, the sample was centrifuged at 3700 RPM and the resulting supernatant was
added to a 39 ml Beckman Opti Seal centrifuge tube. Using a peristaltic pump, a
30   discontinuous gradient of iodixinol (OptiPrep, Nycomed) was added to the tube in 4
layers (60, 40, 25, and 15 % iodixinol). The 15% iodixinol was dissolved in 1 M
NaCl/1X TD. One times TD buffer is 1 X PBS, 1 mM MgCl₂, 5 mM KCl. The 25%
and 40% iodixinols were dissolved in 1X TD. The 60% iodixinol was dissolved in

25

water. Phenol red was included in the 60% and 25% iodixinol to distinguish the layers. The tubes were heat-sealed, placed in a Beckman 70 Ti rotor, and centrifuged at 69000 RPM for 1 hr at 18°C. The AAV was then removed (by pulling the 2nd layer from the bottom of the tube and the interphase between the 1st and 2nd layers),

5   and added to a heparin (Sigma) affinity column. After washing with 1 X TD buffer and eluting with 15 ml of 1 X TD/1 M NaCl, the sample was concentrated in Millipore Biomax 100 Ultrafree-15 units. Prior to adding the sample, the concentrators were coated with 2% rat serum/lactated Ringer's solution (Baxter) overnight at 4°C. The concentrators were then rinsed twice with lactated Ringer's

10   solution and then spun for 2 min at 2700 RPM. The sample was then added and concentrated to 1 ml, before adding 9 ml of lactated Ringer's solution. This 10 ml volume was again concentrated to 1 ml and then 9 ml of lactated Ringer's solution was added. The 10 ml volume was concentrated to 200 µl, which was removed and placed in a siliconized microcentrifuge tube. The concentrator was then rinsed

15   several times with another 200 µl of lactated Ringer's solution, which was then added to the resulting 400 µl stock of rAAV.

Recombinant AAVs were titered for total particles by a previously described method (Zolotukhin et al., 1999). A 4 µl aliquot of the virus stock was treated with DNAse

20   (Boehringer Mannheim) for 1 hr at 37°C, followed by addition of proteinase K buffer (which denatures the DNAse) and treatment with proteinase K (Boehringer Mannheim) for 1 hr at 37°C, in order to obtain only the encapsidated DNA. Ten times DNAse buffer is 50 mM Tris HCl pH 7.5, 10 mM MgCl and 10X proteinase K buffer is 10 mM Tris HCl pH 8.0, 10 mM EDTA, and 10% SDS. The sample was

25   then phenol: chloroform extracted twice and chloroform extracted once before being precipitated with sodium acetate and ethanol. The pellet was dissolved in 40 µl of water (a 1:10 dilution of the original aliquot). The sample was then quantitated for copies of rAAV DNA using a quantitative-competitive PCR assay. This assay utilizes an internal control of known quantity. The same PCR primers amplify both the

30   internal control and the viral DNA. The small size difference in the PCR product derived from the internal control (GFP DNA with a 100 bp deletion) vs. the full length PCR product from the virus allows for titering the particles of rAAV by

titrating increasing amounts of the internal control into this reaction. Titers for the

AAVs that were used were 1×10^{13} particles per ml.

I. Subjects and stereotaxic surgery

5   Male Sprague-Dawley rats (3 months old) were anesthetized with cocktail made up of

3 ml xylazine (20 mg/ml), 3 ml ketamine (100 mg/ml), and 1 ml acepromazine (10

mg/ml) administered intramuscularly at a dose of 0.5-0.7 ml/kg. The injection

coordinates for the SN were −5.3 mm bregma, 2.3 mm medial-lateral, 7.8 mm.

dorsal-ventral (Paxinos and Watson, 1986). Virus stocks were injected through a 27

10   ga. cannula connected via 26 ga. I.D. polyethylene tubing to a 10 μl Hamilton syringe

mounted to a CMA/100 microinjection pump. The pump delivered 3 μl over 30 min,

and the needle remained in place at the injection site for 1 additional min. The

cannula was removed slowly (over 2 min), and the skin was sutured and the animal

was placed on a heating pad until it began to recover from the surgery, before being

15   returned to their individual cages. All animal care and procedures were in accordance

with institutional IACUC and NIH guidelines.

Immunohistochemistry

Anesthetized animals were perfused with 100 ml of cold phosphate-buffered

· 20   saline (PBS), followed by 400 ml of cold 4% paraformaldehyde in PBS. The brain

was removed and equilibrated in a cryoprotectant solution of 30% sucrose/PBS and

stored at 4°C. Coronal sections (30 or 50 μm for qualitative or stereological

assessments, respectively) were cut on a sliding microtome with freezing stage.

Antigen detection was conducted on free-floating sections by incubation in a blocking

25   solution (2% goat serum/0.3% Triton X-100/ PBS) for 1 hr at room temperature,

followed by primary antibody incubation for 2 hr at room temperature on a shaking

platform. Prior to blocking, endogenous peroxidase was quenched by incubation in

0.5% H_2O_2/PBS for 10 min. Primary antibodies and the dilutions used in this study

were: GFP (Molecular Probes polyclonal IgG fraction, 1:1000); α-syn (NACP-98

30   polyclonal, 1:500); TH (Chemicon polyclonal, 1:500); glial fibrillary acidic protein

(GFAP, Chemicon, 1:500). The sections were washed in PBS, and incubated with

biotinylated goat anti-rabbit (DAKO, 1:500) secondary antibody for 1 hr at room

temperature. The sections were washed with PBS and labeled with horseradish

peroxidase (HRP)-conjugated Extravidin (Sigma, 1:1000) for 30 min at room
temperature. Development of tissue labeled with HRP was conducted with a solution
of 0.67 mg diaminobenzidine (Sigma), 0.13 μl of 30% H_2O_2 per ml of 80 mM sodium
acetate buffer containing 8 mM imidazole and 2% $NiSO_4$. For fluorescent labeling,

5 TRITC-conjugated goat anti-rabbit secondary antibody (Sigma, 1:400) followed the
primary antibody. Nuclear counterstaining was performed by incubating the sections
for 10 min in DAPI (Sigma, 1 μg/ml) followed by PBS washes. Fluorescent samples
were coverslipped using glycerol gelatin mounting media (Sigma).

10 *Stereological estimates of dopamine neurons*
The number of SN pars compacta neurons expressing tyrosine hydroxylase
immunoreactivity was estimated by an unbiased stereological method, the optical
fractionator method of West (1991), using a microscope with a motorized stage. Six
to 8 sections regularly spaced throughout the SN pars compacta structure were

15 analyzed. Fifty μm sections were used for stereology. Optical dissectors were
50x50x15 μm cubes spaced in a systematic random manner 100 μm apart and offset 3
μm from the section surface. The fractionator sampling was optimized to yield ca.
150 counted cells per animal, for error coefficients <0.10.

20 *Motor behaviors*
Animals that were injected with either control or α-syn vector unilaterally to
substantia nigra (6/group) were challenged with *d*-amphetamine (free base, 2 mg/kg in
saline, i.m.) at 6 and 12 months after gene transfer. The amphetamine was injected 20
min prior to placing the animals in the TruScan activity montoring system (Coulbourn

25 Instruments), which was used to measure rotational behavior, the number of 360°
turns in either the clockwise or counter-clockwise direction. Animals that were
injected with either control or α-syn vector bilaterally to substantia nigra (8/group)
were tested for their motor coordination and agility on a rotating rod (Economex,
Colombus Instruments) at intervals of 1, 3, 6, and 12 months after gene transfer. The

30 rod was set at speeds of 12, 24, and 36 RPM and the animals were tested in three trials
at each speed for up to 60 sec, unless they fell off the wheel. Rotational behaviors
and fall latencies for each vector group were compared by ANOVA.

The following subsections describe the several results parameters evaluated in this example.

GFP and α-syn expression in substantia nigra neurons

5   The CBA promoter produced robust expression of GFP native fluorescence in SN neurons and their projections with the control vector, pTR-UF12 AAV, throughout the one-year time course of the study (Fig. 6A). Using the same promoter, the pSyn30 AAV was efficient in expressing α-syn immunoreactivity in midbrain neurons and their projections over this interval as well (Fig. 6C, D). For both the

10   control and the α-syn vectors, the injections transduced a major fraction of the SN pars compacta, from its most rostral to caudal aspects. Dopaminergic neurons were transduced, as GFP and TH (visualized with red fluorescence) co-localized with the control vector and the α-syn vector. These vectors were therefore effective for targeting the SN pars compacta neurons, although additional transgene expression

15   occurred outside of the SN in the midbrain.

Immunoreactivity for α-syn accumulated densely in SN pars compacta neurons (Fig. 6E, F). The intracellular accumulations of α-syn immunoreactivity appeared in clumps (Fig. 6E) and also with ring-like cytoplasmic aggregates (Fig. 6F), although

20   classic singular, circular Lewy bodies were not apparent. In preliminary studies, the α-syn accumulation in cells was not co-detected with ubiquitin antibody or thioflavin S at time points of one or six months (not shown, assays conducted by Dr. D. Dickson, Mayo Clinic, Jacksonville, FL), suggesting that the build up of α-syn protein was not ubiquitinated and not formed into β-pleated sheets. The α-syn

25   antibody labeled cell bodies and processes only in samples in which the α-syn vector was injected and no perikaryal or neuritic structure was detected in non-transduced tissues, uninjected animals, or control vector-injected animals. In control samples, the non species-specific antibody (NACP-98) stained the rat brain neuropil more strongly in some areas such as the SN pars reticulata (Fig. 6B) and striatal gray matter (not

30   shown), although no resolvable structures were seen unless the α-syn vector was injected. Neither the control GFP AAV vector nor the α-syn AAV vector injections led to expression in astrocytes or astrogliosis as monitored by counterstaining transduced tissue with the GFAP antibody.

Lewy-like axon morphology

The anterograde labeling of transduced cells with GFP was useful to view the change

in neuronal morphology induced by α-syn gene transfer. GFP-expressing

5   nigrostriatal axons could be seen medial to the SN and in the striatum (Fig. 7A, B)

from either the control or the α-syn vector. Nigrostriatal axons from control CBA

promoter vector group always showed uniform axon thickness with characteristic

small varicosities, as reported previously with a neuron-specific enolase (NSE)

promoter vector (Klein *et al.*, 1999a). After α-syn gene transfer, transduced axons

10   showed a dramatic shift to heterogeneous morphology and thickened axons, and

characteristic spherical swellings (Fig. 7C-H). The swollen spheroids, often larger

than the size of cell bodies, were never observed in controls. The spheroids were 20-

40 μm in diameter and did not stain with the nuclear marker, DAPI. Comparing Figs.

7A & 7C, GFP was expressed in axons in the gray matter of the striatum for either

15   vector group (large, dark circular areas are striatal white matter). More GFP was

found in striatal axons in the control vector group than in the α-syn vector group,

where GFP was bicistronically expressed. Immunofluorescence for α-syn efficiently

labeled striatal axons and large varicosities when ectopic α-syn was expressed (Fig.

7E, F), although in all other control samples, labeling for α-syn in the striatum did not

20   show any morphological structures and only neuropil staining. The morphological

pattern of the α-syn immunoreactivity in the striatum after α-syn gene transfer

included fine thread-like axons with large aggregated clumps (Fig. 7F). Figs. 7G &

7H show co-localization of α-syn and GFP in the α-syn vector group.

25   *Loss of SN dopamine neurons*

One year after injecting either the control or the α-syn vector into the SN, TH-

immunoreactive neuron profiles in the SN were assessed. Expression of GFP in the

control group did not appear to alter the density of dopaminergic neurons in this

region compared to age-matched, uninjected subjects (Fig. 8A, B). The numbers of

30   SN dopamine neurons were estimated for each of the two vector groups. The

stereological assessments revealed a 53% loss in the α-syn group. The number of

TH-immunoreactive neurons in the SN pars compacta on one side of the brain was

estimated to be 8075 ± 835 (n=7) in animals that received control vector, and 3825 ±

378 (n=8) in animals that received the α-syn vector (different from control, $p<0.0005$, t-test).

Motor behavior tests

5   Animals expressing α-syn in their SN were compared with controls for two types of motor behaviors. In one paradigm, animals were injected unilaterally with either control or α-syn vector (6/group) and tested for amphetamine-stimulated rotational bias, which is observed towards the lesioned side if there is a large enough loss of dopamine neurons. In another paradigm, animals (8/group) were injected bilaterally

10   and tested for their ability to walk on a rotating rod set at several speeds, which tests locomotor coordination and agility. The animals were tested at intervals between 1 and 12 months after gene transfer with no robust changes observed relative to control with either test. However, there was a trend that approximated statistical significance at several of the intervals for α-syn treated rats to circle towards the side of the α-syn

15   vector injections, and also for α-syn rats to fall off the rotating rod at shorter times compared to controls (p values between 0.05 and 0.10).

DISCUSSION of Example 8:

20   The most striking aspect of the results of Example 8 is the fact that the SGT of α-syn resulted in a 53% loss of dopaminergic cells in the SN in the study period. The loss of dopaminergic cells in the SN region is a key characterisitic of PD. These results indicate the suitability of SGT in specific brain regions of test animals for model systems to evaluate the progression of neurological diseases under a variety of

25   controlled conditions.

The somatic gene transfer of Example 8 also was used to evaluate other aspects of PD: cytoplasmic accumulation of α-syn, Lewy-like neurites, and locomotor behavior. The results with the CBA promoter demonstrate the ability of an appropriate promoter

30   to effectively target the SN pars compacta dopaminergic neurons with AAV gene transfer and also to express genes in that region for long intervals using neuron-specific promoters. This is consistent with other studies (Klein *et al.*, 1998; Klein *et al.*, 1999a; Klein *et al.*, 1999b; Paterna *et al.*, 2000). Those studies found the NSE

promoter vectors to result in neuron-specific transgene expression, which is what was found herein with the CBA promoter. While the present results are consistent with others that vectors based on AAV-2 are limited for infecting glia (Bartlett *et al.*, 1998), expression in astrocytes has been seen using the cell type-specific GFAP

5 promoter in this vector serotype (Peel and Klein, 2000).

The perikaryal immunoreactivity for α-syn densely filled the cytoplasm in clumps including apparent ring formations. Although the presence of classic, singular Lewy inclusion bodies was not detected, this is similar to what was found in transgenic mice

10 expressing the same form of α-syn (Kahle *et al.*, 2000). Without being bound to specific theories or explanations for the lack of the presence of classic, singular Lewy inclusion bodies in the results of this example, some factors that may mitigate against the formation of such classic Lewy inclusion bodies in this example include: the presence of 'normal' rat synuclein protein in addition to the SGT-added human α-syn

15 variant; differences in expression or arrangement of the human α-syn variant in rat neurons; and insufficient time of observation.

Despite this apparent lack of Lewy body inclusions in the cell bodies, the striking loss of dopaminergic cells in the SN and the Lewy-like neuronal processes in the SN and

20 the striatum indicate that the present invention does provide a suitable model for the study of neurological diseases.

With regard to the Lewy-like neuronal processes, the α-syn gene transfer resulted in a shift from uniform nigrostriatal axon morphology in controls to heterogeneous

25 morphology characterized by thread-like to sausage- and balloon-like morphologies. These are comparable to samples of Lewy neurite pathology in human PD (Haass and Kahle, 2000; van der Putten *et al.*, 2000) and Lewy-like neurite pathology in transgenic mice (Masliah *et al.*, 2000; van der Putten *et al.*, 2000; Kahle *et al.*, 2000). This structural plasticity occurred at the earliest time point tested, one month after

30 gene transfer, and persisted throughout the one year time-course of the study. These data demonstrate that the induction of Lewy-like neuron pathology in adults by mutant α-syn does not require expression during development.

The apparent lack of Lewy bodies in this model allowed us to ask whether these inclusion bodies are a prerequisite for cell loss. SN pars compacta dopamine neurons degenerated over the course of the study, establishing this approach as an animal model of PD. The lack of cell loss in the transgenic mice could be due to differences

5   in the levels of α-syn expressed within the dopaminergic neurons, or perhaps to compensatory mechanisms during development that were avoided by genetic manipulation in adults. It is also possible that the loss of TH immunoreactivity we observed involved down-regulation of TH levels in intact neurons. This possibility can be addressed by injecting a retrogradelabel such as fluorogold into the striatum

10 prior to gene transfer.
Despite the substantial loss of TH immunoreactivity, motor behavior was not significantly impaired. Although 50% losses of dopamine neurons can be sufficient to induce amphetamine–stimulated turning behavior after 6-OHDA lesions in rats (Hefti et al., 1980), it is believed that greater losses are required to induce symptoms

15 in human PD. This difference may be due to the fact that 6-OHDA lesions are typically mapped to the SN sub-region topographically related to the injection site, whereas in PD, the entire SN pars compacta undergoes degeneration. In this model, the spread of the gene transfer affected essentially the entire SN pars compacta. It is therefore possible that for this reason, this genetic model is more similar to human PD

20 than the 6-OHDA lesioning model.

It also is noted that researchers of PD have observed that behavioral effects are not observed until 50 percent or 80 percent of dopaminergic cells in the SN are lost (see chapter 5 of Mechanisms of Degeneration and Protection of Dopaminergic System,

25 pages 105-106, and The absolute number of nerve cells in substantia nigra in normal subjects and in patients with Parkinson's disease estimated with an unbiased stereological method, J. of Neurology, Neurosurgery, and Psychiatry, 1991; 54:30-33). In the rat model, statistically significant behavioral differences may not be consistently observed until greater than the 53 percent loss of dopaminergic cells

30 observed in this example, or without a higher number of subjects observed.

Also, it is noted that some SN dopaminergic neurons expressing α-syn remained at the conclusion of the study, perhaps due to sub-threshold expression levels for

toxicity. This hypothesis could be tested by applying higher doses than the one used and potentially by studying dose-response curves of α-syn vector on dopaminergic cell loss to address whether there is more than one population with differential sensitivities to α-syn. In addition to dose-modification, greater expression levels

5   could be accomplished via more efficient vector systems, for example by incorporating the woodchuck hepatitis post-transcriptional regulatory element (Loeb *et al.*, Paterna *et al.*, 2000), or potentially with other AAV serotypes (Davidson *et al.*, 2000).

10   Alternatively, the survival of some substantia nigra neurons expressing after one year could be due to differences in their processing of the human α-syn. It is hypothesized that oligomeric, protofibrillar α-syn may be the toxic form (Conway *et al.*, 2000; Rochet *et al.*, 2000) and perhaps the surviving cells are able to fibrillize α-syn. There could have been a protective effect in the cells expressing endogenous α-syn and one

15   approach could involve down-regulating rat α-syn with a species-specific ribozyme to potentially block this action. Other differences in the surviving cells could be their ability to ubiquitinate and degrade α-syn, as mutations in the E3 ubiquitin ligase, parkin, are linked to familial PD (Shimura *et al.*, 2001). The model described here should be convenient for combination experiments studying the role of α-syn and

20   other genes in the ubiquitin-proteasome pathway that are linked to PD. These approaches will hopefully help to uncover why dopamine neurons are selectively vulnerable during PD pathogenesis as well as lead towards novel gene therapy strategies for PD.

25   Together, these results demonstrate that alpha-synuclein overexpression in a brain region associated with Parkinson's disease causes a behavioral deficit and cellular morphology typical of the disease. This study demonstrates the utility of this somatic transgene approach for modeling because more classic, much slower, mouse transgenic approaches are still attempting to demonstrate these phenomena.

30

EXAMPLE 9

An experiment is envisioned to either enhance or block the toxicity induced by the SGT introduction of a-syn of Example 8. A sufficient number of test organisms are pre-treated with desired environmental stressors at selected concentrations. One example of an environmental stressor is the neurotoxin 6-hydroxydopamine at sub-

5   threshold levels of detection of behavioral deficits. Also, a sufficient number of these organisms are pre-treated with desired nutrient regimes at selected concentrations. One example of a desired nutrient regime is a standard feed supplemented with high level of an antioxidant that targets brain tissue. Also, a sufficient number of these organisms are pre-treated with one or more desired pharmacologically active

10   substances.

The organisms are then treated with the α-syn mutant via SGT according to the method of Example 8. Organisms that were not pre-treated are then provided with treatments (the post-treatment groups), and selected organisms are given treatments

15   before and after the SGT. Some experimental treatments constitute combinations of nutrient regime(s), pharmacologically active substance(s), and/or environmental stressor(s).

At appropriate times selected organisms are selected for behavioral testing and

20   sacrifice for histology. It is expected that the results of this experiment will provide meaningful information regarding which nutrient regimes and pharmacologically active substances are beneficial to improve the condition of the test organisms with regard to the PD symptoms, and which environmental stressors are deleterious and/or their effects are partially or completely counteracted by one or a combination of

25   nutrient regimes and/or pharmacologically active substances.

This experiment is viewed to meet key objectives of the present invention.

It should be understood that the examples and embodiments described herein are for

30   illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

The teachings of all patents and publications cited throughout this specification are incorporated by reference in their entirety to the extent not inconsistent with the teachings herein.

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REFERENCES

BARTLETT, J. S., SAMULSKI, R. J., MCCOWN, T. J. (1998) Selective and rapid uptake of adeno-associated virus type 2 in brain. Hum Gene Ther. **9**, 1181-1186.

5   CONWAY, K.A., LEE S.-J., ROCHET, J.-C., DING, T.T., WILLIAMSON, R.E., LANSBURY, JR., P.T. (2000) Acceleration of oligomerization, not fibrillization, is a shared property of both a-synuclein mutations linked to early-onset Parkinson's disease: Implications for pathogenesis and therapy. Proc. Natl. Acad. Sci. USA **97**, 571-576.

10   DALY, T.M., OKUYAMA, T., VOGLER, C., HASKINS, M.E., MUZYCZKA, N., and SANDS, M.S. (1999). Neonatal intramuscular injection with recombinant adeno-associated virus results in prolonged beta-glucuronidase expression in situ and correction of liver pathology in mucopolysaccharidosis type VII mice. Human Gene Therapy **10**, 85-94.

15   DAVIDSON, B.L., STEIN, C.S., HETH, J.A., MARTINS, I., KOTIN, R.M., DERKSEN, T.A., ZABNER, J., GHODSI, A., CHIORINI, J.A. (2000) Recombinant adeno-associated virus type 2, 4, and 5 vectors: transduction of variant cell types and regions in the mammalian central nervous system. Proc Natl Acad Sci USA **97**, 3428-3432.

20

DICKSON, D.W. (1999). Tau and synuclein and their role in neuropathology. Brain Pathol. **9**, 657-661.

DIRKS, W., WIRTH, M., HAUSER, H. (1993) Dicistronic transcription units for gene
25   expression in mammalian cells. *Gene* **128**, 247-249.

FEANY, M.B., and BENDER, W.W. (2000). A *Drosophila* model of Parkinson's disease. Nature. **404**, 394-398.

30   GRIMM, D., KERN, A., RITTNER, K., KLEINSCHMIDT, J.A. (1998). Novel tools for production and purification of recombinant adenoassociated virus vectors. Hum Gene Ther. **9**, 2745-2760.

HAAS, C., and KAHLE, P.J. (2000). Parkinson's pathology in a fly (letter). Nature **404**, 341-343.

HEFTI, F., MELAMED, E., WURTMAN, R.J. (1980) Partial lesions of the dopaminergic
5   nigrostriatal system in rat brain: biochemical characterization. Brain Res. **195**, 123-137.

KAHLE, P.J., NEUMANN, M., OZMEN, L., MULLER, V., JACOBSEN, H.,
SCHINDZIELORZ, A., OKOCHI, M., LEIMER, U., VAN DER PUTTEN, H., PROBST, A.,
KREMMER, E., KRETZSCHMAR, H.A., and HAASS, C. (2000). Subcellular localization
10   of wild-type and Parkinson's disease-associated mutant alpha-synuclein in human and
transgenic mouse brain. J. Neurosci. **20**, 6365-6373.

KAPLITT, M.G., LEONE, P., SAMULSKI, R.J., XIAO, X., PFAFF, D.W., O'MALLEY,
K.L., and DURING, M.J. (1994). Long-term gene expression and phenotypic correction
15   using adeno-associated virus vectors in the mammalian brain. Nature Genetics, **8**, 148-154.

KLEIN, R.L., MEYER, E.M., PEEL, A.L., ZOLOTUKHIN, S., MEYERS, C.,
MUZYCZKA, N., and KING, M.A. (1998). Neuron-specific transduction in the rat
septohippocampal or nigrostriatal pathway by recombinant adeno-associated virus vectors.
20   Exp. Neurol. **150**, 183-194.

KLEIN, R.L., MCNAMARA, R.K., KING, M.A., LENOX, R.H., MUZYCZKA, N., and.
MEYER, E. M. (1999a). Generation of aberrant sprouting in the adult rat brain by GAP-43
somatic gene transfer. Brain Res. **832**, 136-144.
25

KLEIN, R. L., LEWIS, M.H., MUZYCZKA, N., and MEYER, E.M. (1999b). Prevention of
6-hydroxydopamine-induced rotational behavior by BDNF somatic gene transfer. Brain Res.
847, 314-320.

30   KLEIN, R.L., MANDEL, R.J., and MUZYCZKA, N. (2000). Adeno-associated virus vector-
mediated gene transfer to somatic cells in the central nervous system. In, Advances in Virus
Res. Vol. **55**. K. Maramorosch, F.A. Murphy, A.J. Shatkin, J.C. Glorioso, eds. (Academic
Press, San Diego, CA) pp. 507-528.

●

KRÜGER, R., KUHN, W., MÜLLER, T., WOITALLA, D., GRAEBER, M., KOSEL, S., PRZUNTEK, H., EPPLEN, J.T., SCHOLS, L., and REISS, O. (1998). Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat. Genet. **18**, 106-108.

5

LEE, V.M. and TROJANOWSKI, J.Q. (1999). Neurodegenerative tauopathies: Human disease and transgenic mouse models. Neuron **24**, 507-510.

LOEB, J.E., CORDIER, W.S., HARRIS, M.E., WEITZMAN, M.D., and HOPE, T.J. (1999)

10   Enhanced expression of transgenes from adeno-associated virus vectors with the woodchuck hepatitis virus posttranscriptional regulatory element: implications for gene therapy. Hum. Gene Ther. **10**, 2295-2305.

MANDEL, R.J., SPRATT, S.K., SNYDER, R.O., and LEFF, S.E. (1997). Midbrain injection

15   of recombinant adeno-associated virus encoding rat glial cell line-deived neurotrophic factor protects nigral neurons in a progressive 6-hydroxydopamine-induced degeneration model of Parkinson's disease in rats. Proc. Natl. Acad. Sci. USA **94**, 14083-14088.

MASLIAH, E., ROCKENSTEIN, E., VEINBERGS, T., MALLORY, M., HASHIMOTO,

20   M., TAKEDA, A., SAGARA, Y., SISK, A., and MUCKE, L. (2000). Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. Science **287**, 1265-1269.

MATSUOKA, Y., VILA, M., LINCON, S., MCCORMACK, A., PICCIZNO, P,

25   LAFRANCOIS, J., YU, X., DICKSON, D., LANGSTON, W.J., MCGOWAN, E., FARRER, M., HARDY, J., DUFF, K., PREZEDBORSKI, S., and DI MONTE, D.A. Lack of nigral pathology in transgenic mice expressing human α-synuclein driven by the tyrosine hydroxylase promoter. Neurobiology of Disease, in press.

30   MCCOWN, T.J., XIAO, X., LI, J., BREESE, G.R., and SAMULSKI, R.J. (1996). Differential and persistent expression patterns of CNS gene transfer by an adeno-associated virus (AAV) vector. Brain Res. **713**, 99-107.

NIWA, H., YAMAMURA, K. and MIYAZAKI, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector, Gene. **108**, 193-9.

PATERNA, J.C., MOCCETTI, T., MURA, A., FELDON, J., and BUELER, H.
(2000). Influence of promoter and WHV post-transcriptional regulatory element on AAV-mediated transgene expression in the rat brain. Gene Therapy **7**, 1304-1311.

PAXINOS, G. and WATSON, C. (1986). The rat brain in stereotaxic coordinates (Academic Press, Sydney).

PEEL, A.L., and KLEIN, R.L. (2000). Adeno-associated virus vectors: activity and applications in the central nervous system. J. Neurosci. Methods **98**, 95-104.

POLYMEROPOULOS, M.H., LAVEDAN, C., LEROY, E., IDE SE, DEHEJIA, A., DUTRA, A., PIKE, B., ROOT, H., RUBENSTEIN, J., BOYER, R., STENROOS, E.S., CHANDRASEKHARAPPA, S., ATHANASSIADOU, A., PAPAPETROPOULOS, T., JOHNSON, W.G., LAZZARINI, A.M., DUVOISIN, R.C., DI IORIO, G., GOLBE, L.I., AND R.L. NUSSBAUM (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science **276**, 2045-2047.

RATHKE-HARTLIEB, S., KAHLE, P.J., NEUMANN, M., OZMEN, L., HAID, S., OKOCHI, M., HAASS, C., and SCHULZ, J.B. (2001). Sensitivity to MPTP is not increased in Parkinson's disease-associated mutant alpha-synuclein transgenic mice. J. Neurochem. **77**, 1181-1184.

ROCHET, J.C., CONWAY, K.A., LANSBURY, JR., P.T. (2000) Inhibition of fibrillization and accumulation of prefibrillar oligomers in mixtures of human and mouse alpha-synuclein. Biochemistry **39**, 10619-10626.

SHIMURA, H., SCHLOSSMACHER, M.G., HATTORI, N., FROSCH, M.P., TROCKENBACKER, A., SCHNEIDER, R., MIZUNO, Y., KOSIK, K.S., SELKOE, D.J.

(2001) Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. Science **293**, 263-269.

TROJANOWSKI, J.Q., and LEE, V.M. (1999). Transgenic models of tauopathies and synucleinopathies. Brain Pathol. **9**, 733-739.

VAN DER PUTTEN, H., WIEDERHOLD, K-H., PROBST, A., BARBIERI, S., MISTL, C., DANNER, S., KAUFFMANN, S., HOFELE, K., SPOOREN, W.P.J.M., RUEGG, M.A., LIN, S., CARONI, P., SOMMER, B., TOLNAY, M., and BILBE, G. (2000), Neurorpathology in mice expressing human α-synuclein. J. Neurosci. **20**, 6021-6029.

WEST, M.J., SLOMIANKA, L., GUNDERSEN, H.J. (1991), Unbiased sterological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. Anat Rec **231**, 482-697.

ZOLOTUKHIN, S., BYRNE, B. J., MASON, E., ZOLOTUKHIN, I., POTTER, M., CHESNUT, K., SUMMERFORD, C., SAMULSKI, R. J. and MUZYCZKA, N. (1999). Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield, Gene Therapy. **6**, 973-985.

WHAT IS CLAIMED IS:

1 1.   A method for producing a non-human animal model of a human disease which
2   comprises transferring at least one aberrant form of at least one gene known to be
3   associated with said disease in humans into appropriate tissue of a living non-human
4   animal under conditions which result in the expression of said at least one aberrant
5   gene, wherein said transferring does not require the modification of the germ-line of
6   said living animal.

1 2.   The method according to claim 1 wherein said human disease is a
2   neurodegenerative disease.

1 3.   The method according to claim 2 wherein said human disease is selected from
2   the group consisting of Alzheimer's Disease, Parkinson's Disease, and Huntington's
3   Disease.

1 4.   The method according to claim 3 wherein said at least one gene is an aberrant
2   form of tau.

1 5.   The method according to claim 3 wherein said aberrant form of tau is P301L,
2   associated with "fronto-temporal dementia with Parkinson's linked to chromosome 17
3   (FTDP-17)".

1 6.   The method according to claim 3 wherein said at least one gene is an aberrant
2   form of alpha-synuclein.

1 7.   The method according to claim 6 wherein said aberrant form of alpha-
2   synuclein is mutant α-synuclein (A30P), associated with Parkinson's Disease.

1 8.   The method according to claim 3 wherein said at least one gene is a mutant
2   amyloid precursor protein (APP), a mutant presenilin-1 (PS1), or combinations
3   thereof, associated with Alzheimer's Disease.

1 9.   The method according to claim 1 which comprises identifying a combination

2 of genes relevant to a particular human pathology and somatically transferring

3 combinations of said genes into tissues appropriate to said particular human pathology

4 in a non-human animal model appropriate to said human pathology.

5 10.   The method according to claim 1 comprising:

6   (a) controlling the location to which the genes are transferred, that is spatially

7   controlling gene expression of the transferred genes, in the non-human animal

8   model to which said at least one gene is transferred;

9   (b) controlling the temporal effects of transferred genes at specific times in the

10   development of otherwise normal organisms, or in the development of

11   organisms in which germline modifications have previously been made, by

12   selecting the time at which said transferred genes are introduced into said

13   organism, or by controlling the time of expression of said transferred genes;

14   (c) evaluating the effects of expression of combinations of multiple transgenes,

15   which in a germline transgenic non-human animal would be difficult if not

16   impossible to achieve due to diseases which might prevent the animal model

17   from maturing to the age-appropriate state for modeling onset of a particular,

18   complex human disease;

19   (d) increasing the rate for analyzing multiple genes which contribute to complex,

20   multifactorial human diseases by transferring more than a single gene into an

21   appropriate non-human animal model for said disease;

22   (e) testing pharmaceutical agents for their ability to ameliorate specific diseases .

23   induced in said non-human animal model;

24   (f) studying specific human pathologies induced in said non-human animal model

25   by inducing said pathology in said animal model by transferring said at least

26   one gene into said animal model;

27   (g) supplementing an existing germline transgenic model with additional

28   somatically provided gene products to modulate the transgenic model;

29   (h) creating a disease condition in an otherwise healthy animal; and

30 combinations of (a) –(h).

1 11.   A non-human animal produced by the method of claim 1.

1   12.   A pharmaceutical identified through testing of pharmaceutical compounds

2   using the non-human animal produced according to claim 11.

1   13.   A method for inducing neurofibrillary tangles in the brain of a non-human

2   animal which comprises injecting into the brain of said animal an effective amount of

3   a gene expression construct encoding tau, alpha-synuclein, presenilin-1, amyloid

4   precursor protein, IL6, or a combination thereof.

1   14.   A non-human animal produced according to the method of claim 13.

1   15.   A method for inducing behavioral changes in a non-human animal model

2   which comprises somatic administration of at least one gene directly to the brain of

3   said non-human animal, wherein said at least one gene is associated with a human

4   neurodegenerative disease.

1   16.   The method according to claim 1 wherein said at least one aberrant form of

2   said at least one gene is transferred by means of an adeno-associated virus.

1   17. A method of identifying a pharmacologically active substance that slows, reduces,

2   reverses or eliminates the adverse effects of a degenerative neurological disease,

3   comprising:

4   a.   transferring at least one aberrant form of at least one gene known to be

5   associated with said disease in humans into appropriate tissue of a

6   living non-human animal under conditions which result in the

7   expression of said at least one aberrant gene, wherein said transferring

8   does not require the modification of the germ-line of said living

9   animal;

10   b.   repeating step "a" for a sufficient number of living non-human animals

11   to evaluate a desired number of pharmacologically active substances at

12   a desired number of concentrations, combinations, administration

13   regimes, and evaluation schedules;

14   c.   exposing a specified number of said sufficient number of living non-

15   human animals of step "b" to a specific pharmacologically active

16   substance at a desired number of concentrations and administration

17   regimes, and optionally repeating this exposing for each of a desired
18   number of additional specific pharmacologically active substance(s);
19   and
20   d.   evaluating said specific pharmacologically active substance(s) of step
21   "c";
22 wherein said evaluating provides information that identifies at least one
23 pharmacologically active substance that slows, reduces or eliminates the adverse
24 effects of a degenerative neurological disease.

1   18.   The method of claim 17, wherein the step "c" exposing begins prior to said
2   transferring of at least one gene of steps "a" and "b".

1   19.   The method of claim 17, wherein the step "c" exposing begins prior to and
2   continues after said transferring of at least one gene of steps "a" and "b".
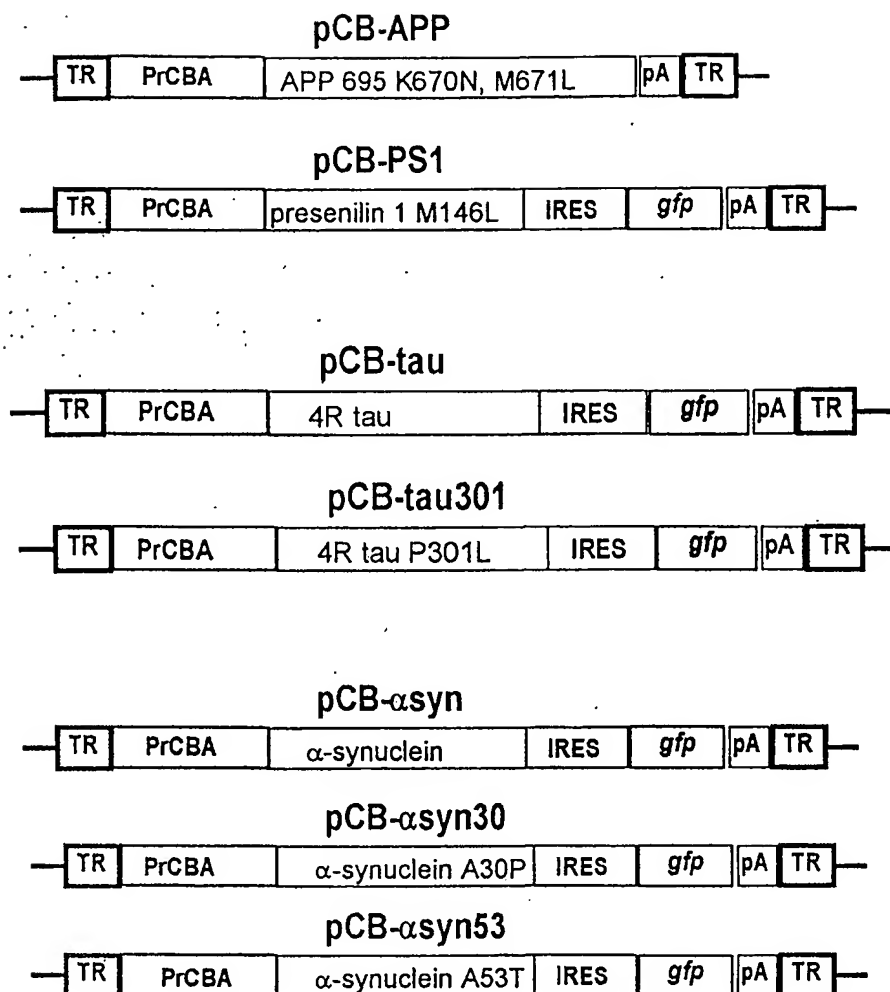
1   20.   The method of claim 17, wherein the step "c" exposing begins after said
2   transferring of at least one gene of steps "a" and "b".

1   21.   The method of claim 17, wherein the effects of at least one nutrient regime
2   and/or the effects of at least one environmental stressor also are evaluated.

1   22. A method of identifying a nutrient regime that slows, reduces, reverses or
2   eliminates the adverse effects of a degenerative neurological disease, comprising:
3   a.   transferring at least one aberrant form of at least one gene known to be
4   associated with said disease in humans into appropriate tissue of a
5   living non-human animal under conditions which result in the
6   expression of said at least one aberrant gene, wherein said transferring
7   does not require the modification of the germ-line of said living
8   animal;
9   b.   repeating step "a" for a sufficient number of living non-human animals
10   to evaluate a desired number of pharmacologically active substances at
11   a desired number of concentrations, combinations, administration
12   regimes, and evaluation schedules;

13   c. exposing a specified number of said sufficient number of living non-

14   human animals of step "b" to a specific pharmacologically active

15   substance at a desired number of concentrations and administration

16   regimes, and optionally repeating this exposing for each of a desired

17   number of additional specific pharmacologically active substance(s);

18   and

19   d. evaluating said specific pharmacologically active substance(s) of step

20   "c";

21 wherein said evaluating provides information that identifies at least one

22 pharmacologically active substance that slows, reduces or eliminates the adverse

23 effects of a degenerative neurological disease.

1 23. A method of identifying an environmental inducer that aggravates, induces, or

2 otherwise contributes to the adverse effects of a degenerative neurological disease,

3 comprising:

4   a. transferring at least one aberrant form of at least one gene known to be

5   associated with said disease in humans into appropriate tissue of a

6   living non-human animal under conditions which result in the

7   expression of said at least one aberrant gene, wherein said transferring

8   does not require the modification of the germ-line of said living

9   animal;

10   b. repeating step "a" for a sufficient number of living non-human animals

11   to evaluate a desired number of pharmacologically active substances at

12   a desired number of concentrations, combinations, administration

13   regimes, and evaluation schedules;

14   c. exposing a specified number of said sufficient number of living non-

15   human animals of step "b" to a specific environmental inducer at a

16   desired number of concentrations and administration regimes, and

17   optionally repeating this exposing for each of a desired number of

18   additional specific environmental inducer(s); and

19   d. evaluating said specific environmental inducer(s) of step "c";

20 wherein said evaluating provides information that identifies at least one

21 environmental inducer that aggravates, induces, or otherwise contributes to the

22 adverse effects of a degenerative neurological disease.

46

pCB-APP

| TR | PrCBA | APP 695 K670N, M671L | pA | TR |

pCB-PS1

| TR | PrCBA | presenilin 1 M146L | IRES | gfp | pA | TR |

pCB-tau

| TR | PrCBA | 4R tau | IRES | gfp | pA | TR |

pCB-tau301

| TR | PrCBA | 4R tau P301L | IRES | gfp | pA | TR |

pCB-αsyn

| TR | PrCBA | α-synuclein | IRES | gfp | pA | TR |

pCB-αsyn30

| TR | PrCBA | α-synuclein A30P | IRES | gfp | pA | TR |

pCB-αsyn53

| TR | PrCBA | α-synuclein A53T | IRES | gfp | pA | TR |

FIGURE. 1. DNA Constructs used in this application. The schematic diagrams represent the expression cassettes which are packaged into the recombinant adeno-associated virus (AAV) vectors. Abbreviations: TR, AAV terminal repeats; PrCBA, cytomegalovirus/chicken beta-actin hybrid promoter; IRES, internal ribosome entry sequence which allows for bicistronic expression of two transgenes; gfp, green fluorescent protein; pA, poly adenylation sequence. Human DNA sequences to model neurodegenerative diseases: APP, amyloid precursor protein mutant form linked to Alzheimer's disease; presenilin 1 mutant form linked to Alzheimer's disease; tau wild type and mutant form linked to fronto-temporal dementia with parkinsonism linked to chromosome 17; alpha-synuclein wild type and mutant forms linked to Parkinson's disease.
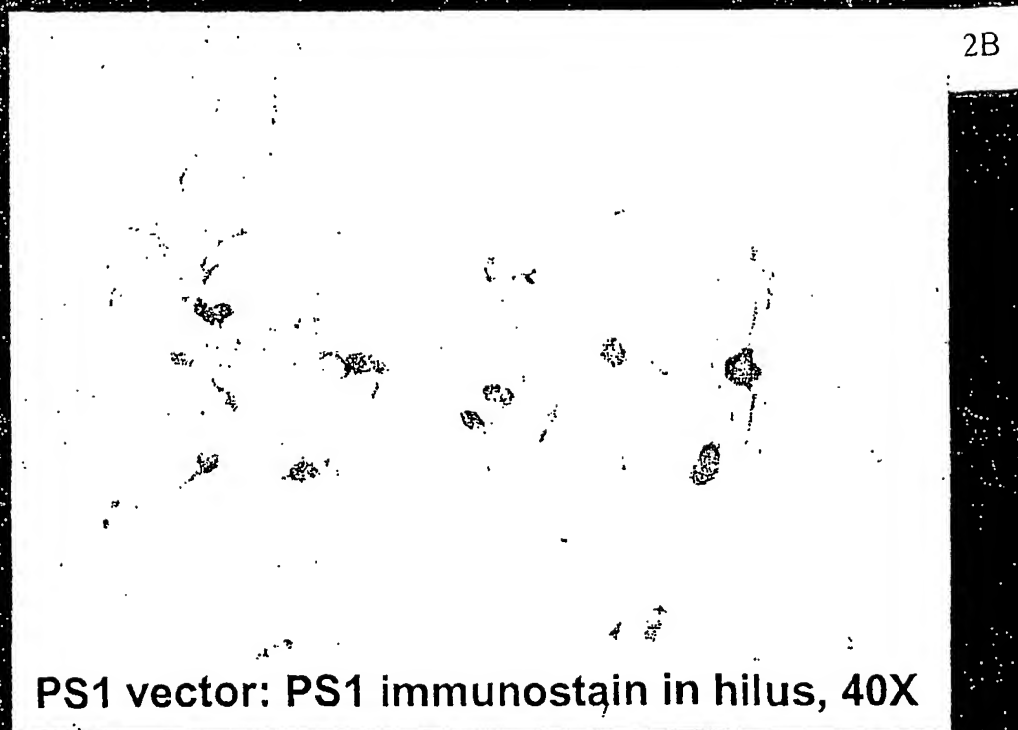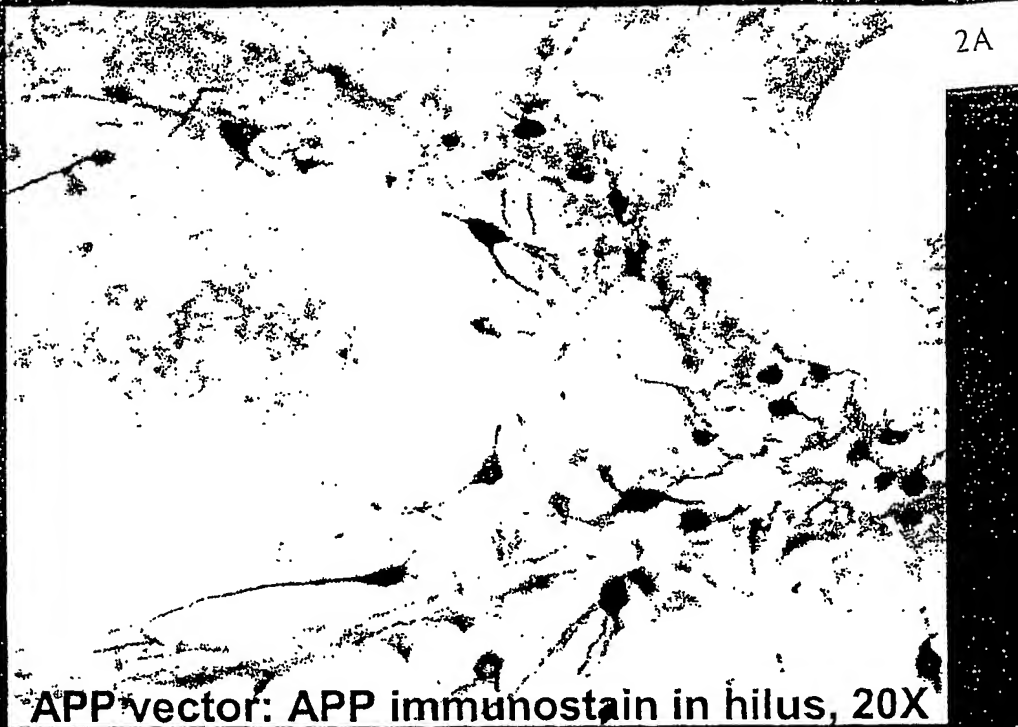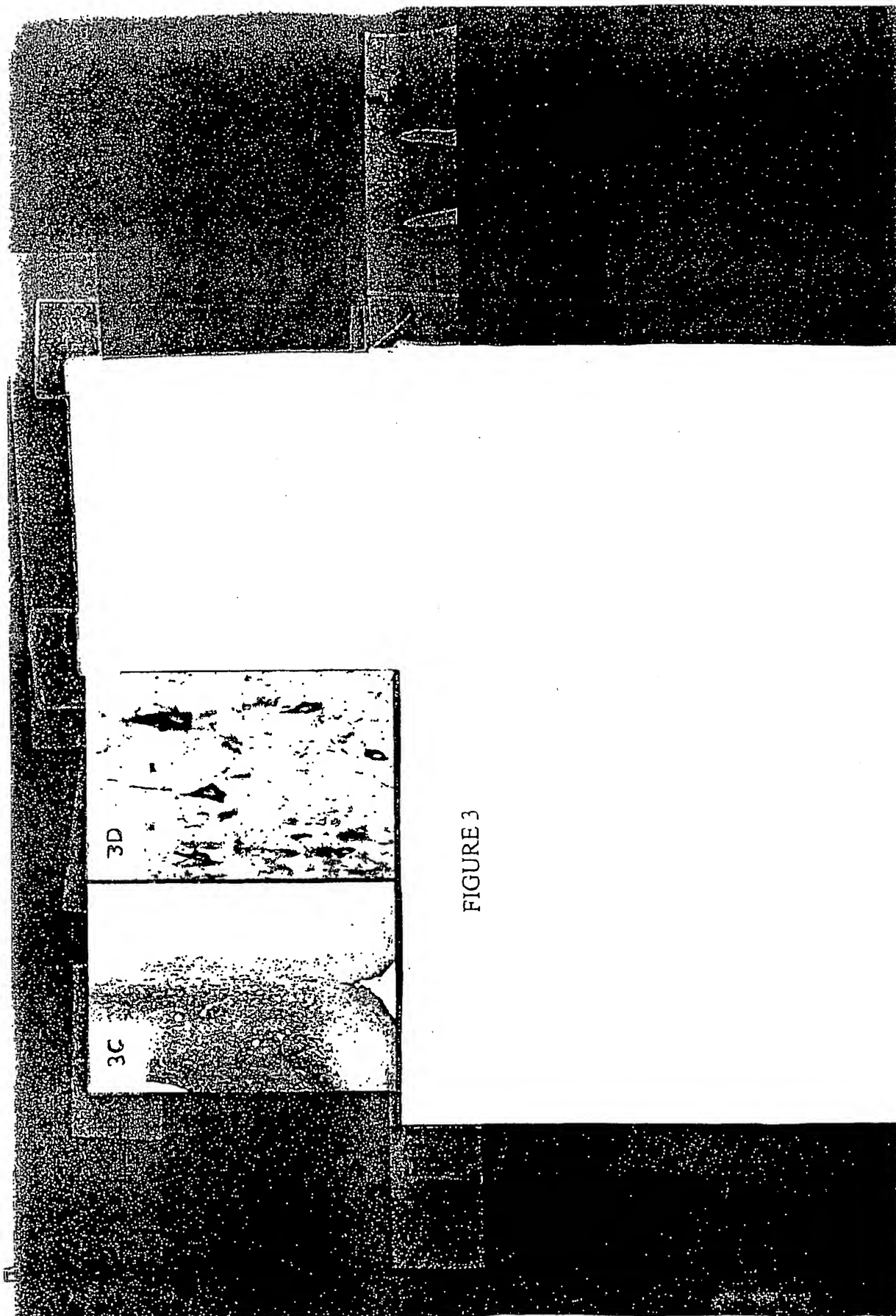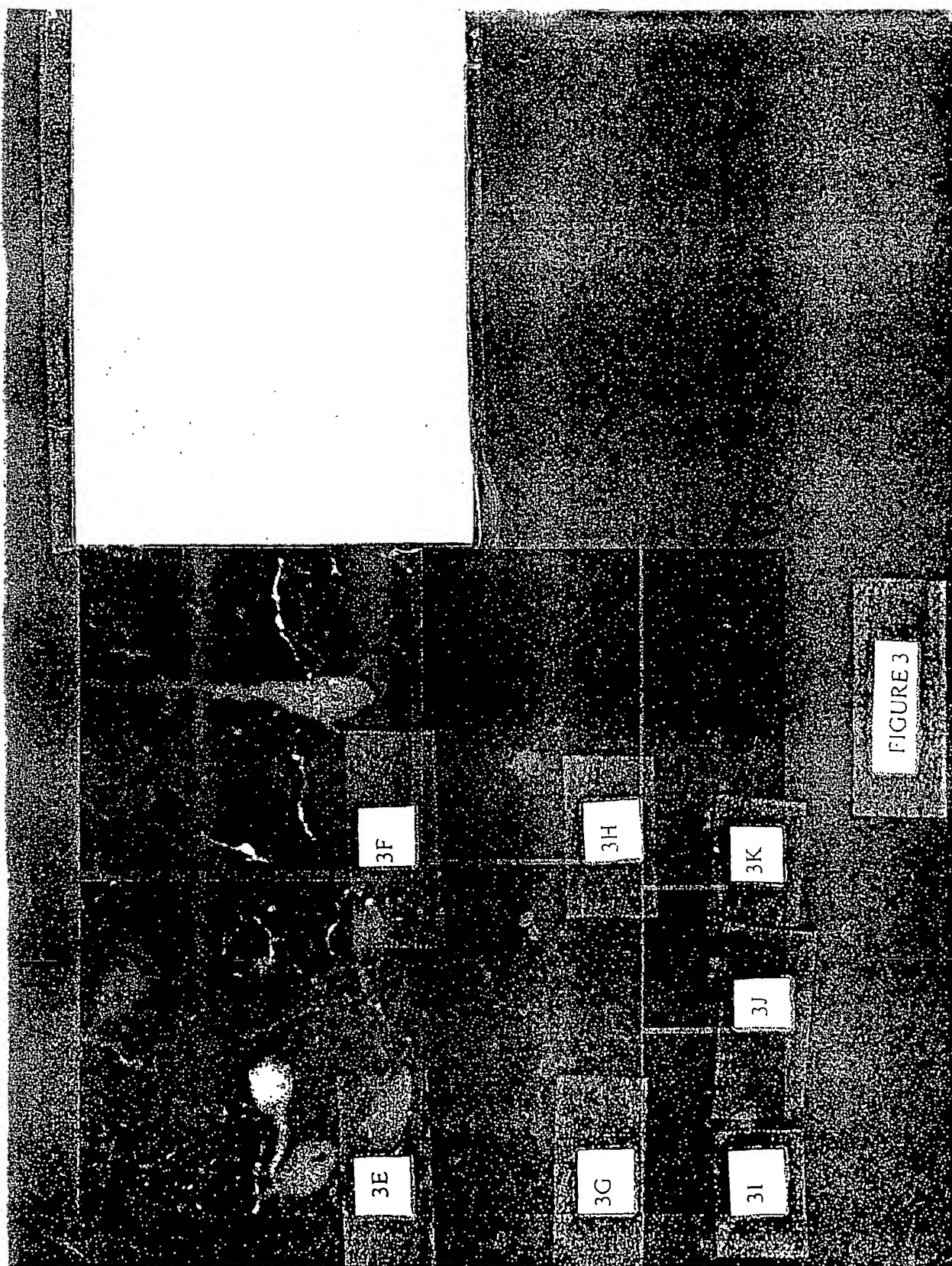
2A

APP vector: APP immunostain in hilus, 20X

2B

PS1 vector: PS1 immunostain in hilus, 40X

FIGURE 2

red: tau
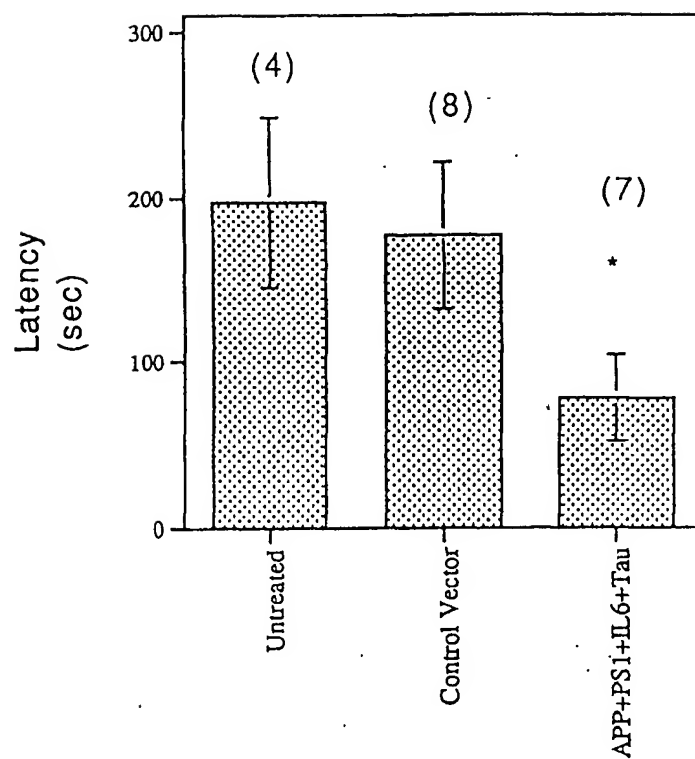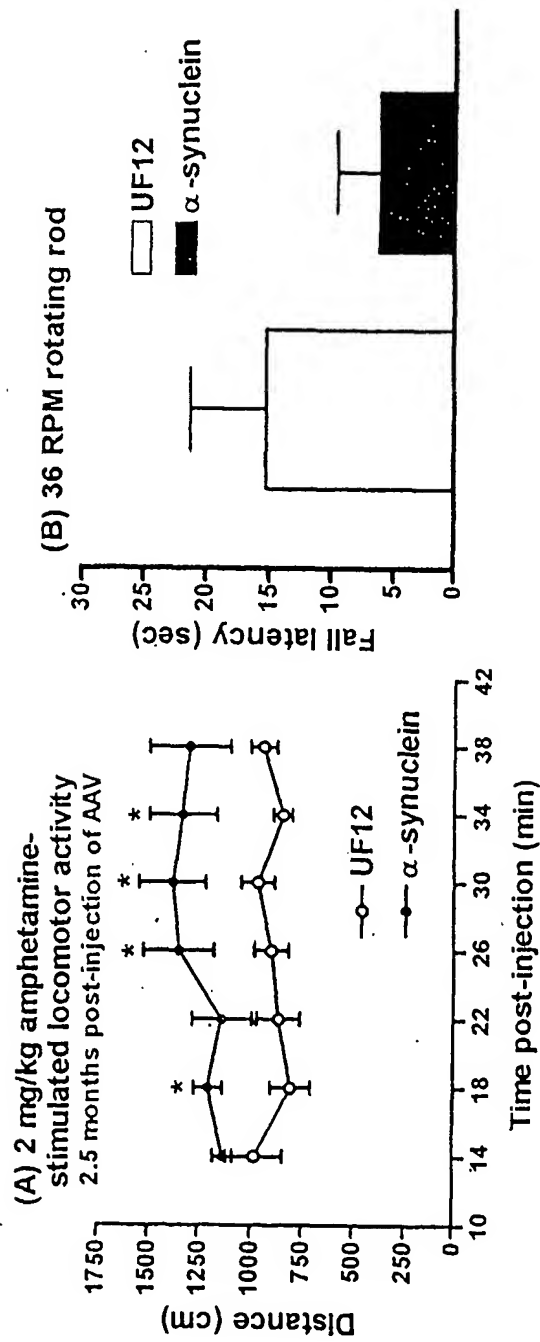green: GFP
blue: DAPI

3A

3B
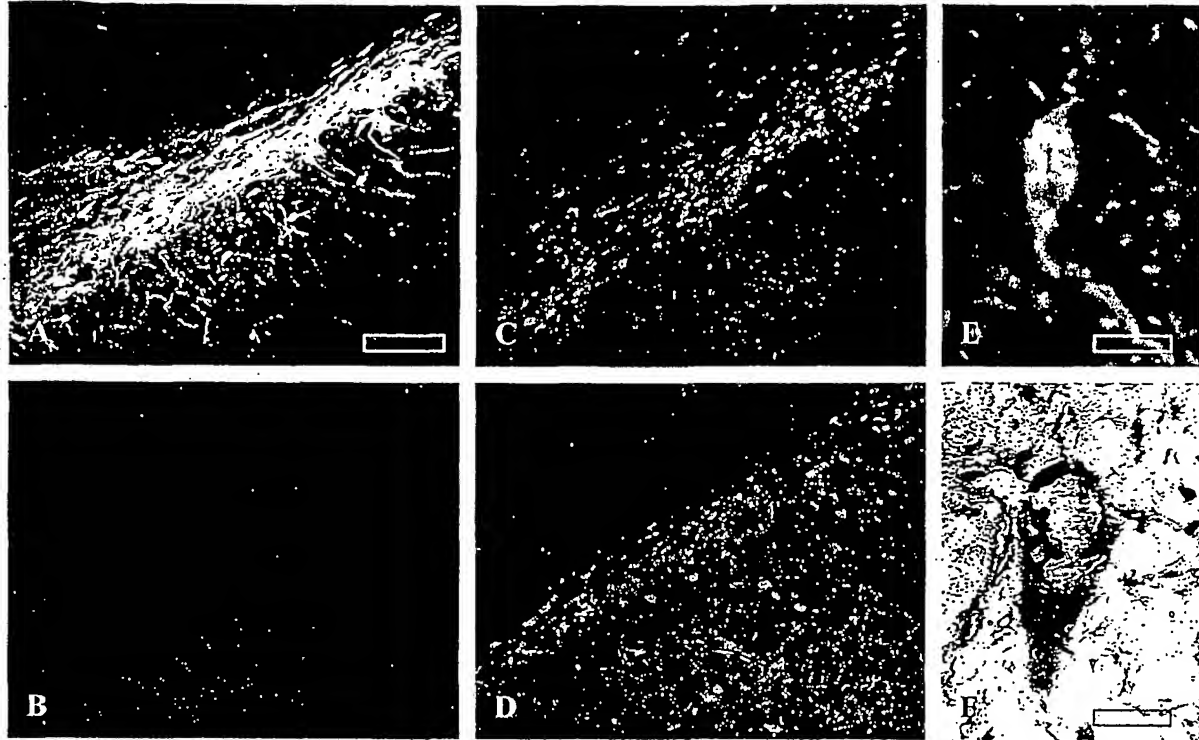
filamentous tau

FIGURE 3

3D

3C

FIGURE 3

FIGURE 3

FIGURE 4

FIGURE 5

Fig. 6

Fig. 7

Fig. 8